Oxidative shielding and the cost of reproduction in the blue tit *Cyanistes caeruleus*

Submitted by

Miranda Alicia Rosanna Walter

to the University of Exeter as a dissertation for the degree of

Masters by Research in Biological Sciences

October 2017

I certify that all material in this dissertation which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) ……

Abstract

The cost of reproduction is considered to be a cornerstone of life history theory. However, the physiological mechanism that underpins the cost of reproductionremains unclear. Recently, considerable interest has been given to oxidative stress as a functional mediator of the cost of reproduction. Empirical studies aiming to test the oxidative cost of reproduction have so far yielded equivocal results. Recently, a new hypothesis was presented, offering a framework for future study design. The 'oxidative shielding' hypothesis proposes that the cost of reproduction is mediated by dual impacts of maternally-derived oxidative damage on mothers and offspring, and that mothers may be selected to shield offspring from such damage transmitted during reproduction. In this landscape-scale study on populations of wild blue tits *Cyanistes caeruleus* I have tested the oxidative cost of reproduction within the framework proposed by the 'oxidative shielding' hypothesis. I present data indicating a longitudinal change in oxidative status from pre-laying to clutch completion suggestive of oxidative shielding within breeding females. Additionally, oxidative stress in females is seen to impose a constraint on clutch size, and the transmission of oxidative damage from mother to egg was found to have negative implications for offspring survival. Lastly, examination of the association between oxidative stress and antioxidants within yolk and plasma suggests a protective role of vitamin E in yolk, but no association with oxidative damage in plasma. These findings provide preliminary support for the 'oxidative shielding' hypothesis and highlight areas of focus for future research into the oxidative cost of reproduction.

Table of Contents

Chapter 1: General Introduction……. 6

Chapter 2: Maternal effects on egg oxidative state and offspring survival in wild blue tits (*Cyanistes caeruleus*)……... 16

Abstract…… 17

Introduction…….. 18

Methods…… 23

Results…….. 29

Discussion…… 40

Chapter 3:The consequences of vitamin E provisioning on the susceptibility of lipids to peroxidation in egg yolk of the blue tit *Cyanistes caeruleus*…….....48

Abstract……..49

Introduction……...50

Methods…….54

Results……...57

Discussion…….62

Chapter 4: General Discussion…….....67

References:……..76

List of Tables and Figures

Chapter 1

Figure 1.1. Schematic relationship between reproduction and oxidative stress as proposed by the 'oxidative shielding' hypothesis…….11

Chapter 2

Figure 2.1. The change in MDA concentrations between Julian bleed date at pre-laying and clutch completion with the change in individual birds displayed…….31

Figure 2.2. The change in α-tocopherol concentrations between Julian bleed date at pre-laying and clutch completion…….31

Figure 2.3. Relationship between plasma MDA concentration pre-laying and at clutch completion with 95% confidence intervals……32

Figure 2.4. The predicted relationship between female plasma α-tocopherol concentration at pre-laying and clutch size with 95% confidence intervals……34

Figure 2.5. The predicted relationship between female plasma MDA concentration at pre-laying and clutch size with 95% confidence intervals……..34

Figure 1.6. Relationship between the absolute change in plasma MDA and the concentration of yolk MDA with 95% confidence intervals……...35

Table 2.1.The relationships between pre-laying oxidative state at pre-laying and egg production (2016 only)……36

Figure 2.7. The predicted relationship between yolk MDA concentrations and fledging success with 95% confidence intervals……38

Table 2.2. The relationships between yolk MDA and α-tocopherol concentrations on hatching and fledging success…….39

Chapter 3

Table 3.1. Therelationship betweenMDA and α-tocopherol and carotenoids in yolk between years…….59

Figure3.1. The relationship between yolk MDA and α-tocopherolconcentrations in 2010 and 2015 with 95% confidence intervals……..59

Figure3.2. Yolk MDA concentrations in each treatment group with standard error……...60

Table 3.2. The influence of antioxidants on the susceptibility of yolk to lipid peroxidation. All main effects and significant interactions from GLMM minimum adequate model reported……..……..61

Figure 3.3. The predicted relationship between yolk concentrations of α-tocopherol and the absolute change in yolk concentrations of MDA after treatment with FeSO4 with 95% confidence intervals…….61

Chapter 1

General Introduction

Life history traits are associated with the critical stages of an animal's life trajectory, including their rate of development, age at first breeding, how many reproductive events they undertake and how long they live. Perhaps the most fundamental life history trade-off is that between reproduction and longevity. Life history theory determines that current reproductive investment incurs a cost onthe future reproductive success and longevity of the individual (Stearns 1992). This cornerstone of life history theory underpins reproductive strategies across species and is critical for evolution by natural selection. While there is a wealth of research into the nature of reproductive trade-offs across taxa, the underlying physiological mechanisms that determine them are yet to be fully understood (Selman et al. 2012; Stier et al. 2012; Speakman & Garratt 2014; Isaksson et al. 2011; Metcalfe & Monaghan 2013; Speakman et al. 2015; Blount et al. 2016).

A leading theory as to the currency of these life history trade-offs is oxidative stress. Oxidative stress is thought to be one important physiological cause of senescence (Beckman & Ames 1998; Halliwell 1991; Harman 1956; Halliwell 1999) and is thought to constrain life history trade-offs such as that between reproduction and longevity. Theoretically, reproduction is costly due to the elevation in metabolic rate which is predicted to increase the production of reactive oxygen species (ROS), exposing the breeding individual to greater risk of oxidative stress. ROS are a group of highly reactive oxygen-containing molecules that are produced as by-products primarily via oxidation-reduction

reactions during ATP synthesis in the mitochondria (Barja 2007). Although the majority of free oxygen is converted to water, a small amount is converted into one of several types of ROS (Dowling & Simmons 2009). These free radicals abstract particular atoms from cells, catalysing reaction chains and destabilising macromolecules including DNA, lipids and proteins, ultimately causing oxidative damage to cells (Beckman & Ames 1998; Dowling & Simmons 2009). While antioxidant systems have naturally evolved to combat oxidative damage, during times of elevated free radical production or suppressed antioxidant defence, the antioxidant system is overwhelmed and oxidative damage occurs (i.e. oxidative stress; (Hulbert et al. 2007)). However, the nature of the relationship between energy generation and oxidative damage is still largely unknown (Speakman & Garratt 2014; Selman et al. 2012) and is variable among species who have evolved different mechanisms to deal with oxidative stress (Balaban et al. 2005).

The mitigation of oxidative damage is primarily controlled by the antioxidant system, which has been widely examined across taxa. Cells contain a repertoire of antioxidant defences, including both enzymatic and non-enzymatic antioxidants, which act as pro-oxidant scavengers or work to repair and maintain cellular machinery (Beckman & Ames 1998). Due to the intrinsic association between oxidative stress and the antioxidant system, many early studies testing the oxidative cost of reproduction used antioxidants to measure oxidative stress. These early findings produced some support for an oxidative cost of reproduction (Alonso-Alvarez et al. 2004; Bertrand et al. 2006; Wiersma et al. 2004). However, using only antioxidants to determine oxidative stress is now deemed insufficient and potentially misleadinggiven that levels of antioxidants can either increase or decrease under an oxidative

attack,depending on whether the body's defences are depleted due to high levels of ROS or are downregulated in times of low oxidative stress (Selman et al. 2012; Metcalfe & Monaghan 2013). Indeed, currently there is no specific assay to measure oxidative stress and the pathway from ROS generation to oxidative damage is undoubtedly complex (Selman et al. 2012). What is crucial to oxidative stress measurements are markers of oxidative damage in proteins (e.g. carbonyls and thiols), lipids (e.g. malondialdehyde (MDA) and hydroperoxides), and DNA (e.g. 8-hydroxy-20-deoxyguanosine, 8-hydroxyguanosine, and the Comet assay) (Selman et al. 2012). Repair mechanisms (e.g. proteasome activity) may also be useful to measure the efficiency of damage limitation which may be important to the outcome of oxidative stress. Antioxidant assays (e.g. superoxide dismutase, vitamin E, total antioxidant capacity (TAC)) have been used abundantly, but they do not provide a measure of the outcome of oxidative stress and are often non-specific. Several recent reviews have indicated that studies thus far have failed to measure appropriate indicators of oxidative stress(Selman et al. 2012; Stier et al. 2012; Isaksson et al. 2011; Metcalfe & Monaghan 2013; Speakman & Garratt 2014; Blount et al. 2016). They suggest that the choice of assays used to measure oxidative stress are crucial to the validity and relevance of results. They also suggest thatin order to gain a true representation of the oxidative status within an individual, it is essential to combine markers of oxidative damage, antioxidant assays specific to the type of damage marker used, and if possible a measurement of repair mechanisms.

More recent empirical studies using various measures of oxidative stress, including direct markers of damage, have yielded equivocal results. Some have provided supporting evidence for an oxidative cost of reproduction, showing

increases in oxidative damage in breeders (Alonso-Álvarez et al. 2010; Bergeron et al. 2011; Fletcher et al. 2012; Stier et al. 2012; Xu et al. 2014; Olsson et al. 2009). Others, however, found an opposing pattern and reduced levels of oxidative damage associated with reproduction (Garratt et al. 2012; Garratt et al. 2013; Ołdakowski et al. 2012; Yang et al. 2013; Costantini, Casasole, et al. 2014), or no evidence of any increase in oxidative stress with reproductive effort (Nussey et al. 2009). However, recent reviews of the evidence so far suggest that the variability in findings is due to limitations in study design (Isaksson et al. 2011; Metcalfe & Monaghan 2013; Selman et al. 2012; Speakman & Garratt 2014; Blount et al. 2016; Stier et al. 2012). For example, Bergeron *et al.* (2011) concede that individual age, reproductive status and environmental conditions all contribute to the oxidative outcome of reproduction and must therefore be considered in future experimental design. Similarly, Stier*et al.* (2012) found that oxidative damage only acted as a constraint specifically at the pre-breeding period of reproduction, highlighting the need for longitudinal study design over the reproductive process. In contrast, both Fletcher *et al.* (2012) and Xu *et al.* (2014) measured oxidative status post-breeding only, and found breeders to have lower antioxidant protection and elevated levels of a marker of oxidative damage (protein carbonyls) when compared with pre-breeding state (Fletcher et al. 2012) and non-reproducing counterparts (Xu et al. 2014). However, Xu *et al.* (2014) acknowledge that post-breeding sampling limits the interpretation of the results to consequent reproductive costs, rather than oxidative constraints which may determine investment initially. Directly opposing results to these have been seen in house mice *Musmusculusdomesticus*(Garratt *et al.* 2011, 2012)and bank voles *Myodes glareolus*(Ołdakowski *et al.* 2012), showing reduced levels

of oxidative damage in several markers during lactation, even under experimentally enlarged litter sizes. Similar results have been shown in breeding canaries *Serinuscanaria,* which show a decrease in plasma oxidative damage (reactive oxygen metabolites and protein carbonyls) compared with non-breeding individuals (Costantini*et al.* 2014). However, results from Garratt *et al.* (2011, 2012) and Ołdakowski *et al.* (2012) also suggested that oxidative damage was positively correlated with fecundity, supporting results from Stier*et al.* (2012) and Olsson *et al.* (2009) that oxidative damage may act as a constraint on reproductive investment. Others go further to suggest that females employ strategies to mitigate oxidative costs at specific periods (Garratt et al. 2011, 2012, King et al 2013, Yang et al. 2013), complicating the relationship between oxidative stress and reproduction and demanding exact measures of oxidative stress at specific reproductive stages.Furthermore, it has been suggested that studies to date have failed to successfully manipulate reproductive effort over a lifetime, which is an important step as an individual's reproductive investment at one reproductive event may in fact be determined by past or future events, ultimately governing lifetime reproductive success (Metcalfe & Monaghan 2013; Visser & Lessells 2001). It is clear, therefore, that precise methodologies, including carefully planned longitudinal studies and specific measures of oxidative stress, are critical for gaining a true representation of the oxidative costs of reproduction (Isaksson et al. 2011; Metcalfe & Monaghan 2013; Selman et al. 2012; Speakman & Garratt 2014; Stier et al. 2012).

In response to the equivocal support found for the oxidative cost of reproduction, the 'oxidative shielding' hypothesis (Blount et al. 2016) was recently proposed as an explanation. It provides a framework created from

detailed analysis of previous studies that aims to explain the variation in results observed so far and sets out specific methodologies for future research to consider. The hypothesis suggests that “the transition into the reproductive state triggers a pre-emptive reduction in levels of oxidative damage in many tissues, in order to shield mothers, and in particular their gametes and developing offspring, from harm caused by an inevitable increase in oxidative damage resulting from expenditure of reproductive effort” (Blount et al. 2016).

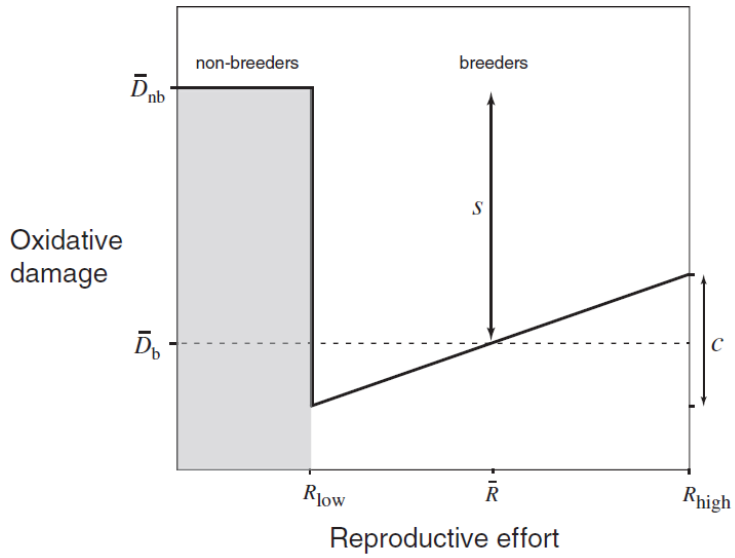

Figure 1.1. Schematic relationship between reproduction and oxidative stress as proposed by the ‘oxidative shielding’ hypothesis (Blount et al. 2016)

Acknowledging this hypothesis sheds new light on the potential for longitudinal changes in oxidative state over the reproductive process and may allow previously inconclusive or conflicting data to be reconsidered within the theory’s framework. It is now apparent that both the timing and method of measuring oxidative stress is imperative to the results achieved, as both reproductive stage

and choice of tissue and damage marker show fluctuation in oxidative state over the reproductive process.

The ‘oxidative shielding’ hypothesis is based on the foundation that there is a risk of maternal transfer of oxidative stress, accumulated during the reproductive process, to offspring, and this has caused a specific ‘shielding’ period to evolve during reproduction. This highlights a secondary oxidative consequence of reproduction whereby parents not only incur oxidative damage themselves, but also pass this damage on to offspring. Indeed, the idea that maternally derived oxidative stress may significantly influence offspring fitness suggests that the transmission of oxidatively damaged components to offspring has a role as a maternal effect, similar to that of maternally derived antioxidants but with potentially deleterious consequences. Despite the expansive field of research in maternal effects, investigation into potentially negative naturally occurring maternal effects such as oxidative stress is largely unknown. Several studies on birds investigating the impacts of oxidative stress on parental provisioning ability have found results suggesting a sex specific maternal effect of environment matching on oxidative stress in fledglings (De Coster et al. 2012; Merkling et al. 2017) and increases in oxidative damage during provisioning (Cram et al. 2015; Costantini, Bonisoli-alquati, et al. 2014). Similarly, sibling competition has been seen to influence oxidative stress in wild kestrelchicks *Falco tinnunculustinnunculus*(Costantini et al. 2006) and also in experimentally enlarged broods of zebra finches*Taeniopygiaguttata*(Alonso-Alvarez 2006). However, although these findings suggest oxidative stress may be influential as a maternal effect, so far there have been no studies to directly test whether there are significant consequences of oxidative damage when passed to offspring. For example, markers of oxidative damage (lipid peroxidation) have

been found in the milk of dairy cattle (Bouwstra et al. 2008; Rizzo et al. 2013; Suriyasathaporn et al. 2009), the eggs of fish (Mourente et al. 1999), and also in avian egg yolk (Mohiti-Asli et al. 2008), but the consequences of this has not been directly measured.

The concept that oxidative stress may impose maternal effects on offspring is worth consideration if it does indeed determine reproductive investment and select for 'oxidative shielding' in mothers. Maternal effects comprise a class of phenotypic effects whereby maternal phenotype directly effects the phenotype of her offspring and is unrelated to the offspring's genotype (Bernardo 1996). Eggs offer an ideal study system for maternal effects research, being a self-contained package with all the nutrients required for the developing embryo (Bernardo 1996). Eggs also provide a snap-shot representation of the physiological state of the mother at the point of laying and are therefore useful in maternal effects research, such as the examination of the transgenerational impacts of oxidative stress on offspring as suggested by the 'oxidative shielding' hypothesis. In this study we used assays of lipid peroxidation (MDA) and antioxidant concentration (vitamin E and carotenoids) to measure the oxidative status of breeding female blue tits *Cyanistes caeruleus* and their eggs and chicks. Blue tits are abundant in deciduous woodlands and nest in boxes, providing an ideal study system within a naturally evolved context to study the oxidative cost of reproduction.

This study aims to address some of the key elements put forward by the 'oxidative shielding' hypothesis. In chapter 2 we look at the longitudinal changes in the oxidative status of breeding females over the reproductive period, using plasma assays of MDA and vitamin E taken both before the breeding season

and directly after clutch completion to examine whether there is a reduction in oxidative stress from the pre-breeding state to offspring production. These chosen assays gained both a measure of lipid peroxidation and levels of antioxidant defence, providing a balanced overview of the oxidative status of the tissues sampled as suggested in recent reviews (Selman et al. 2012; Metcalfe & Monaghan 2013; Blount et al. 2016). The use of plasma samples rather than tissue samples enabled longitudinal analysis of individuals, a crucial aspect within this study. We also investigated the transmission and influence of oxidative damage and antioxidants from mother to egg, removing one egg in each clutch for analysis of yolk tissue. To standardise egg removal, the last laid egg in each clutch was used for analysis. Clutches were followed to completion and survival was assessed and analysed for any relationships between adult and egg oxidative status and offspring fitness.

In chapter 3 we examine the relationship between oxidative damage and antioxidant activity to further understand the relationship between these systems and the role of both oxidative damage and antioxidants in determining oxidative stress status and consequent reproductive decisions. We subjected yolk to an oxidative challenge (FeSO4) *ex-vivo* and assessed the susceptibility of yolk lipid to peroxidation depending on the level of antioxidants (vitamin E and carotenoids) present in the yolk. We aim to examine the consequences of transmitting harmful products of oxidative damage to eggs and how mothers might protect offspring during such a vulnerable period.

Lastly, in chapter 4 we discuss the findings in this body of work and how it can inform our understanding of the 'oxidative shielding' hypothesis. We also suggest areas of interest for future study and discuss the relevance of this work

in the broader field of life history research, including how such information can help in understanding how species might be affected by environmental change.

Chapter 2

Maternal effects on egg oxidative state and offspring survival in wild blue tits (*Cyanistes caeruleus*)

Miranda A.R. Walter[1],Kate E. Plummer[2]& Jonathan D. Blount[1]

[1]Centre for Ecology and Conservation, College of Life and Environmental Sciences, University of Exeter, Penryn Campus, Cornwall, TR10 9FE, UK

[2]British Trust for Ornithology, Daphne du Maurier Building, University of Exeter, Penryn Campus, Penryn, Cornwall, TR10 9FE, UK

Authors for correspondence: marw201@exeter.ac.uk; j.d.blount@exeter.ac.uk

Abstract

Knowledge of the causes and consequences of oxidative stress during reproduction is critical for understanding the physiological costs that underpin life history trade-offs. Oxidative damage has been proposed as a key mechanism mediating such trade-offs, but empirical data on the association between reproduction and oxidative damage are equivocal. It was recently suggested that these opposing patterns can be reconciled if mothers pre-emptively reduce levels of oxidative damage on transition to breeding in order to protect themselves, and their physiologically dependent offspring, from harm caused by the inevitable increase in oxidative damage that ensues through the expenditure of reproductive effort (the 'oxidative shielding' hypothesis). Here, we provide longitudinal data on changes in maternal oxidative damage and antioxidant defences from pre-breeding through egg laying in wild blue tits (*Cyanistes caeruleus*). We also examine the relationship between maternal oxidative state and egg phenotype, and the consequences for chick survival.

Introduction

It is well established that reproduction and lifespan are constrained by trade-offs which prevent their simultaneous increase (Stearns 1992). However, the underlying mechanisms remain poorly understood (Isaksson et al. 2011; Metcalfe & Monaghan 2013; Speakman & Garratt 2014; Selman et al. 2012). One proposed mechanism is oxidative stress, which arises if reactive oxygen species (ROS) overwhelm the body's antioxidant defence and repair mechanisms. ROS are highly reactive oxygen-containing molecules produced primarily via oxidation-reduction reactions during ATP synthesis in mitochondria (Dowling & Simmons, 2009; Barja 2007). Physiological processes that are metabolically demanding, such as reproduction, can potentially lead to increased ROS production and consequent damage to important biomolecules such as DNA, lipids and proteins, leading to loss of cell homeostasis and function (Monaghan et al. 2009; Dowling & Simmons 2009; Beckman & Ames 1998; Halliwell 1999). To date, however, evidence that reproduction increases oxidative damage has been equivocal, with some studies showing increased oxidative damage in breeders (Alonso-Álvarez et al. 2010a; Bergeron et al. 2011; Stier et al. 2012; Xu et al. 2014; Fletcher et al. 2012), while other studies have not found evidence of this (Nussey et al. 2009) or have found the opposite pattern (Dowling & Simmons 2009; Monaghan et al. 2009; Garratt et al. 2011; Costantini, Bonisoli-alquati, et al. 2014; Speakman & Garratt 2014; Blount et al. 2016; Garratt et al. 2012; Garratt et al. 2013; Ołdakowski et al. 2012; Yang et al. 2013).

Failure to consistently detect an oxidative cost of reproduction has been attributed to weaknesses in study design, such as a focus on inappropriate

markers of oxidative stress. For example, it is now broadly accepted that measurements of antioxidants are insufficient to infer levels of oxidative stress; markers of oxidative damage are essential (Monaghan et al. 2009; Gutteridge & Halliwell 2000; Speakman & Garratt 2014; Metcalfe & Alonso-Alvarez 2010; Metcalfe & Monaghan 2013; Blount et al. 2016; Selman et al. 2012; Isaksson et al. 2011). Recently, however, Blount *et al*. (2016) used meta-analysis to show that notwithstanding any failures in the design of studies, across species there are in fact significantly reduced levels of oxidative damage in breeding compared to non-breeding females, while paradoxically, there is a positive association between breeding effort (i.e. offspring number) and levels of oxidative damage (Blount et al. 2016). They suggested that these opposing patterns may be explained if mothers pre-emptively reduce levels of oxidative damage on transition to breeding in order to protect themselves, and in particular their physiologically dependent offspring, from harm caused by the inevitable increase in oxidative damage which ensues through the expenditure of reproductive effort (the 'oxidative shielding' hypothesis (Blount et al. 2016)).

The 'oxidative shielding' hypothesis awaits detailed empirical investigation, and in particular data are lacking on the association between oxidative damage in breeding mothers and impacts on the development and survival of their offspring (Blount et al. 2016). Several studies have provided cross-sectional data on assocciations between reproduction and oxidative state (Alonso-Álvarez et al. 2010b; Isaksson 2013; C Isaksson et al. 2011; Nussey et al. 2009; Bize et al. 2008), however, longitudinal data are required in order to examine how oxidative damage levels may change over the course of non-breeding and breeding states and the consequences for offspring (Blount et al. 2016). Such longitudinal monitoring requires minimally invasive sampling, which

tends to restrict researchers to the utilisation of blood rather than organ tissue analysis. Whether blood can provide markers of oxidative state which are representative of the whole organism, or predict fitness outcomes, remains a topic of debate (Blount et al. 2016; Speakman et al. 2016). However, to advance this discussion we need more studies which attempt to link blood markers of oxidative damage and antioxidant defences to the development and survival of offspring, i.e. fitness outcomes (Blount et al. 2016; Speakman et al. 2016).

The avian egg offers an excellent opportunity to study how maternal oxidative state may impact on offspring, since the egg is a self-contained package that provides all of the nutrients required by the developing embryo (Bernardo 1996). Transfer of nutrients and other potentially beneficial compounds such as hormones, antibodies and lysozymes into eggs are examples of maternal effects (Saino et al. 2002; Royle et al. 2001; Tschirren et al. 2014; Muriel et al. 2015; D'Alba et al. 2010; Pihlaja et al. 2006), where maternal experience of the environment can shape offspring phenotype thereby increasing the adaptability and fitness of offspring beyond genetic constraints (Wolf & Wade 2009). Maternal effects can generate a large amount of phenotypic variation among offspring, and are important for selection, potentially providing a platform on which beneficial maternal effects may accumulate (Mausseau, Timothy A. & Fox 1998; Räsänen & Kruuk 2007). However, there is ongoing discussion as to whether maternal effects are necessarily adaptive, or merely a channel by which environmental variation passes through the maternal phenotype to offspring (Marshall & Uller 2007). Indeed, maternal effects may not always direct evolutionary trajectories towards improvement (Räsänen & Kruuk 2007; Tilgar et al. 2016), and may act to enhance maternal fitness while having

detrimental impacts on offspring (Hayward & Wingfield 2004; Rubolini et al. 2005; Groothuis & Schwabl 2008; Groothuis et al. 2005). It is well established from studies of birds that toxins such as heavy metals, polychlorinated biphenyls (PCBs) and organohalogens can be transferred from maternal circulation into egg yolk (Kubota et al. 2002; Burger & Eichhorst 2005; Mora 2003; Verreault et al. 2007; Van den Steen et al. 2009). Moreover, there is evidence in dairy cattle that malondialdehyde (MDA), a marker of lipid peroxidation, is deposited in milk and thereby may be transferred to offspring (Suriyasathaporn et al. 2009; Rizzo et al. 2013). Comparable studies investigating deposition of oxidative damage into eggs are lacking. Such intergenerational impacts of oxidative stress may seem likely, because egg yolk is richly endowed with maternally-derived polyunsaturated fats (Royle et al. 1999) which are especially susceptible to oxidative damage (Surai et al. 1999a; Surai et al. 1996), that serve to provide the developing embryo with oxidisable substrates to meet its energy demands (Blount et al. 2002; Perrins 1996; Blount et al. 2000). Indeed, the fatty acid composition within the eggs of great tits *Parus major*was found to differ between urban and rural habitats, likely due to dietary differences, with eggs from urban habitats containing proportionately lower levels of polyunsaturated fatty acids(Toledo et al. 2016). This variation in fatty acid provisioning had subsequent impacts on embryo development,whichsupports the idea that there are transgenerational impacts resulting from variation in the state of polyunsaturated fatty acids within avian eggs. If oxidative damage markers are found to be transmitted to offspring via the avian egg, the most parsimonious explanation is obligate deposition alike examples in dairy cattle. In general, studying how oxidative stress may influence life histories is complicated by the fact that we often do not know

which tissues to measure, or which markers of oxidative damage are most relevant to fitness (Speakman et al. 2015; Blount et al. 2016; Isaksson et al. 2011). In the case of avian eggs, however, markers of lipid peroxidation in egg yolk are the obvious focus for study.

Here, we investigate changes in plasma oxidative status from pre-breeding through laying to chick rearing in wild blue tits (*Cyanistes caeruleus)*. We also examine associations between levels of antioxidants (α-tocopherol – a major isoform of vitamin E), and a marker of lipid peroxidation (MDA) in maternal circulation and in freshly laid eggs. Vitamin E serves as a major chain-breaking antioxidant, influential in quenching oxyradicals and interrupting sequences of oxidation (Blount et al. 2000; Surai 2000). We hypothesised that high levels of oxidative damage and low levels of antioxidants in circulation just prior to egg production would be associated with reduced reproductive investment, e.g. smaller clutch sizes. The 'oxidative cost of reproduction' hypothesis would be supported if levels of oxidative damage in maternal circulation increased during the course of egg production. On the other hand, the 'oxidative shielding' hypothesis (Blount et al. 2016) would be supported if egg production was associated with decreased plasma levels of oxidative damage. We further hypothesised that there would be a negative correlation between levels of MDA and antioxidants in maternal plasma and yolk, respectively. Finally, we hypothesised that levels of MDA in yolk would have a negative impact on the hatching success and fledging success of chicks.

Methods

Study system

The study was carried out during spring 2010 and 2016 in Cornwall, UK, at four deciduous woodland sites (Grid refs: SW7837, SW7737, SW7537, SW8240) ranging from 9.5 to 15 hectares in area. The predominant tree species were oak (*Quercus*spp.), beech (*Fagussylvatica*), sweet chestnut (*Castaneasativa*) and sycamore (*Acer pseudoplatanus*). Nest boxes were distributed along transects at 25 metre intervals. Nest boxes were monitored for nest building activity from mid-March; upon the initiation of nesting boxes were checked every other day, and once egg laying commenced boxes were examined daily until clutch completion. Newly laid eggs were weighed on a portable balance to 0.01g (ProScale 111, E-scales, Chester, UK), measured using a digital metal calliper (Stainless Steel Electronic Digital VernierCaliper 0-150mm, Simply Bearings Ltd. UK), and marked using a fine point marker pen to indicate laying order.

Clutches were deemed to be complete when two days had elapsed with no further eggs being laid, and incubation had commenced (Perrins 1996). At this point the last-laid egg was removed and stored at -80°c for biochemical analysis, and nests were then left undisturbed until they were next inspected on the expected day of hatching (i.e. after 12 days of incubation (Perrins 1996). Nest abandonment was assumed if eggs were cold to touch and showed no embryo when candled under torchlight. On the day of hatching or the following day, total brood mass was measured to the nearest 0.01g using a portable balance (Pro Scale 111, E-scales, Chester, UK). Brood number and mass were

again measured at days 8±1 and 14±1. At day 8-9 an aluminium identification ring was fitted to the tibio metatarsus under BTO licence.

Adults were caught under BTO licence using spring traps (Amber Electronics Ltd, Daventry, UK) at three specific stages of breeding. Pre-laying catches were conducted during March and early April when birds were roosting just prior to nest building. Pre-laying catches were on average 15±2 days before laying commenced. Laying day was on average Julian day 124±1 day. Females were bled for the second time two days after the last egg was laid (hereafter, 'clutch completion'), and again at day 8 ± 1 day of brood provisioning. The incubation period in blue tits is 12-14 days (Perrins 1979). Thus, there was a minimum interval of 14 days between blood samples from the same individual. Adults were sexed based on the presence (females) or absence (males) of a brood patch, aged (1 year or > 1 year) according to the colour and condition of the primary coverts (Svensson 1992), and then fitted with an individual aluminium identification ring (BTO). Body mass was measured (±0.1g) using an electronic balance (Pro Scale 111, E-scales, Chester, UK), wing length was recorded using a wing rule (±0.5mm) and head-bill length was measured three times using a digital caliper (±0.005mm) and averaged (Digital VernierCaliper 0-150mm, Simply Bearings Ltd. UK).

Blood samples (50-70µl) were collected from the brachial vein under Home Office licence. Blood was syphoned into a heparinised capillary tube (Fisher Scientific UK Ltd.) following venepuncture with a sterile 23 gauge needle (Fisher Scientific UK Ltd.). Samples were stored in a cool box and centrifuged (4 min, 13 x g) at the field site as soon as convenient following collection (HaematoSpin 1400, Hawksley, Surrey, UK) (mean ± SE time between blood sampling and

centrifugation, 57.89 ± 4.05 mins). Plasma was subsequently transferred into a -80 C freezer in the laboratory until biochemical analysis (mean ± SE time between blood sampling and transfer to -80 C storage, 111.82 ± 6.62mins).

Biochemical analyses

Malondialdehyde (MDA)

Plasma and yolk concentrations of MDA were determined by High Performance Liquid Chromatography (HPLC) as described previously (Plummer 2011). In brief, a 10µl aliquot of plasma was vortexed with 10µl butylatedhydroxytoluene (BHT) (0.05% w/v in 95% ethanol), 80µl phosphoric acid solution and 20µl thiobarbituric acid (TBA). Samples were then heated in a dry bath incubator at 100 C for 1 hour. After cooling on ice (5 mins), 50µl *n*-butanol was added and vortexed (20 s) before being centrifuged (4 mins, 12 x g, at 4 C). A 40µl aliquot of the upper MDA-TBA adduct was collected for HPLC analysis. For MDA determination in yolk, samples (40-50 mg) were placed in phosphate buffered saline (PBS) at 5% w/vand then homogenised for 30 sec using a T18 Basic Ultra-Turrax® homogeniser (IKA® England LTD). A 20µl aliquot of homogenate was then transferred to a reaction tube together with 20µl BHT (0.05% w/v in 95% ethanol, EtOH), 40µlTBA, and 160µl phosphoric acid solution, and the mixture was vortexed for 5 sec before being placed in a dry heat bath at 100°C for 1h. Samples were then cooled on ice, 100µl of *n*-butanol was added and samples were vortexed for 20 sec, then centrifuged (3 mins, 12 x g, 4 C). An aliquot (60µl) of the upper phase, containing MDA-TBA adducts, was drawn off for HPLC analysis. Samples (20µl) were injected in an HPLC system (Dionex Corporation, California, USA) fitted with a Hewlett-Packard Hypersil 5µ ODS 100 x 4.6 mm column and a 5µ ODS guard column (Thermo Fisher Scientific

Inc. Massachusettes, USA), maintained at 37C, following Nussey*et al*. (2009). The mobile phase was methanol-buffer (40:60 v/v) at a flow rate of 1 ml min^{-1}, the buffer being 50mM potassium monobasic phosphate adjusted to pH 6.8 using 5M potassium hydroxide. Fluorescence detection (Dionex RF2000) was performed at 515 nm (excitation) and 553 nm (emission). MDA concentrations were calibrated using an external standard of 1,1,3,3-tetraethoxypropane (TEP) serially diluted with 40% ethanol. Yolk MDA concentrations are reported as µg/g, and plasma MDA concentrations as µg/ml.

α-Tocopherol

Antioxidants in blood plasma and yolk were determined as described previously (Agarwal & Chase 2002). A 10µl aliquot of plasma was vortexed (20s) with a 10µl of 5% sodium chloride (NaCl) and 20µl EtOH. Hexane (600µl) was added, and samples were vortexed for another 20s and then centrifuged (4 min, 13 x g, 4 C). Similarly, egg yolk (40-50 mg) was vortexed in 0.7mL 5% NaCl for 5 sec and then homogenised with 1mL EtOH for 20 sec. Hexane (1.5mL) was added and samples were further homogenised for 10 sec, before being centrifuged (4 min, 8 × g, 4 C), and the hexane phase containing then antioxidants drawn off. A further 1.5mL hexane was added for a second extraction and both hexane extracts were then combined. An aliquot (500µl) of hexane was drawn off and dried under gaseous nitrogen, before the residue was re-dissolved by vortexing for 20 s with 150µl dichloromethane (DCM) and 150µl methanol (MeOH). An aliquot (20 µl) of the DCM-MeOH mixture was injected into an HPLC system (Dionex Corporation, California, USA). Separation utilized a 3-µ C_{18} reverse-phase column (15 cm × 4·6 mm) (Spherisorb S30DS2; Phase separations,

Clwyd, UK), with a mobile phase of methanol (HPLC grade)/ water (97 : 3 v/v) at a flow rate of 1.1 mL min^{-1}. Fluorescence detection (Dionex RF2000) was performed at 295 nm (excitation) and 330 nm (emission). The α-tocopherol peak was identified and quantified by comparison with a standard solution of α-tocopherol (T3251 Sigma-Aldrich) in methanol. Plasma α-tocopherol concentrations are reported as μg/ml, and yolk α-tocopherol concentrations as μg/g.

Statistical analyses

All statistical analyses were performed using R studio version 3.3.3 (Copyright© 2017 The R Foundation for Statistical Computing). Data were analysed using general linear models (GLMs) and general/generalized linear mixed effect models (GLMMs) from the lme4 package, with backwards stepwise deletion of non-significant terms based on AIC, starting with the interaction terms, where alpha was set at 0.05.

Blood sampling was conducted during 2016 only, and we attempted to catch all females on three occasions. Forty-four females were bled in total (pre-laying N = 19, clutch completion N = 32, provisioning N = 6). Ten individuals were bled at both pre-laying and clutch completion, and 3 individuals were bled at both clutch completion and provisioning; 1 individual was bled at all three stages. Therefore, since sample sizes were limited for longitudinal analyses, cross-sectional analyses of plasma levels of MDA and α-tocopherol were analysed first to assess any population level changes. These analyses utilised general linear mixed models (GLMM) with sampling stage as a factor (pre-laying, clutch

completion, provisioning), including laying date as a covariate and site as the random term. Similarly, GLMM was used to analyse the longitudinal data for the individuals bled at both pre-laying and clutch completion (MDA N = 10, α-tocopherol N = 8 due to limited plasma volumes).

Due to small sample sizes available for plasma analysis, plasma MDA and α-tocopherol were included as independent variables in interaction with clutch size and laying date in separate mixed models to avoid convergence issues when analysing the relationship between pre-oxidative state and egg production. Egg phenotype, hatching and fledging data were available for 2010 and 2016. Hatching and fledging success from 2010 and 2016 were converted into binary response variables (hatched : unhatched, fledged : unfledged), which also controlled for clutch size, and were tested in a binomial GLMM. The number of chicks to fledge was also tested as a measure of offspring survival. Laying date was included in interaction with yolk MDA and α-tocopherol in all models to control for seasonal variation, and clutch size was also included as a covariate in all models except when the binary response variable was used. Year and laying date by year were included in analysis of the two year data set. Site was included as a random term in all mixed models and individual (bird) identification was also included as a random term in longitudinal analysis. All main effects, and any significant two-way interactions are reported. Values are reported as means ± SE.

Results

1.Changes in plasma levels of oxidative damage between pre-laying, clutch completion, and provisioning.

In the cross-sectional analysis, plasma MDA concentrations did not differ significantly between pre-laying, clutch completion and provisioning (pre-laying: N = 11, 11.78 ± 1.37 µg/ml, clutch completion: N = 32, 12.25 ± 1.17 µg/ml; provisioning: N = 6, 7.86 ± 0.59 µg/ml; GLMM with site as a random factor: stage, χ^2 = 0.00, d.f. = 6, P = 0.99; laying date, χ^2 = 2.01, d.f. = 5, P = 0.16; stage x laying date, NS). Similarly, there was no significant change in plasma MDA between pre-laying and clutch completion in a longitudinal analysis although later laying date was associated with higher levels of plasma MDA (GLMM for repeated measures with individual and site as random factors: stage, χ^2 = 0.82, d.f. = 6, P = 0.37; laying date, χ^2 = 5.08, d.f. = 5, P = 0.025; stage x laying date, NS). This is because changes in plasma MDA between pre-laying and clutch completion showed considerable variation, with MDA increasing in some females over this period, while levels decreased in others. To evaluate these patterns further, pre-laying MDA data were divided into 'low' (n = 5) or 'high' (n = 5) categories depending on whether they fall below or above the population median (11.97 µg/g), and the absolute change in MDA between pre-laying and clutch completion was calculated for each individual. MDA levels differed between 'low' and 'high' females pre-laying (t-test: t = 4.52, d.f. = 8, P = 0.002). While 'high' females showed a decline in MDA to clutch completion, 'low' females showed the opposite pattern (change in MDA: 'high', -

6.87 ± 1.47 µg/ml; ‘low’, 2.42 ± 1.90 µg/ml; t-test: t = -3.88, d.f. = 8, P = 0.047). Thus, at clutch completion there was no significant difference in plasma MDA between the two groups (t = 1.23, d.f. = 8, P = 0.25). Despite large variation in pre-laying MDA levels, by the time of clutch completion females had converged towards a more similar level of plasma MDA.

We checked whether there was any difference in the timing of pre-laying blood sampling between these ‘high’ and ‘low’ females. Indeed, females in the ‘high’ MDA category were bled on average two weeks later than individuals in the ‘low’ category (Julian bleed date: high MDA, 115 ± 3 d, low MDA, 100 ± 5 d; t-test: t = 2.59, d.f. = 8, P = 0.032). Laying date was slightly later for females in the ‘high’ category although the difference was not statistically significant (Julian laying date: high MDA, 126 ± 4 d, low MDA, 117 ± 3 d; t-test: t = 1.90, d.f. = 8, P = 0.095). Thus, the interval between pre-laying blood sampling and the initiation of laying was shorter for ‘high’ category females (‘high’ 12 ± 2 d, ‘low’ 19 ± 4 d). In an analysis when these differences in the timing of sampling were accounted for, the interval between pre-laying blood sampling and initiation of laying had no significant influence on the absolute change in MDA seen in ‘high’ and ‘low’ females (GLMM: pre-laying MDA category, χ^2 = 10.57, d.f. = 4, P = 0.001; pre-laying blood sample to laying date interval, χ^2 = 0.08, d.f. = 5, P = 0.78; pre-laying MDA x interval interaction, NS; Figure 2.1.). Higher plasma levels of MDA pre-laying were associated with higher plasma levels of MDA at clutch completion (GLMM with site as a random factor: χ^2 = 4.17, d.f. = 4, P = 0.041; Figure 2.3).

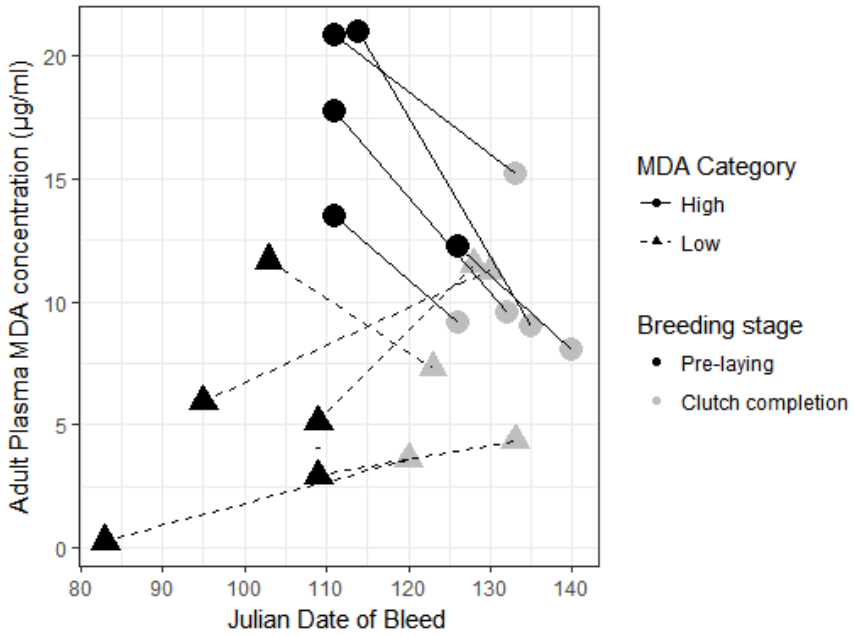

Figure2.1. The change in MDA concentrations between Julian bleed date at pre-laying and clutch completion with the change in individual birds displayed

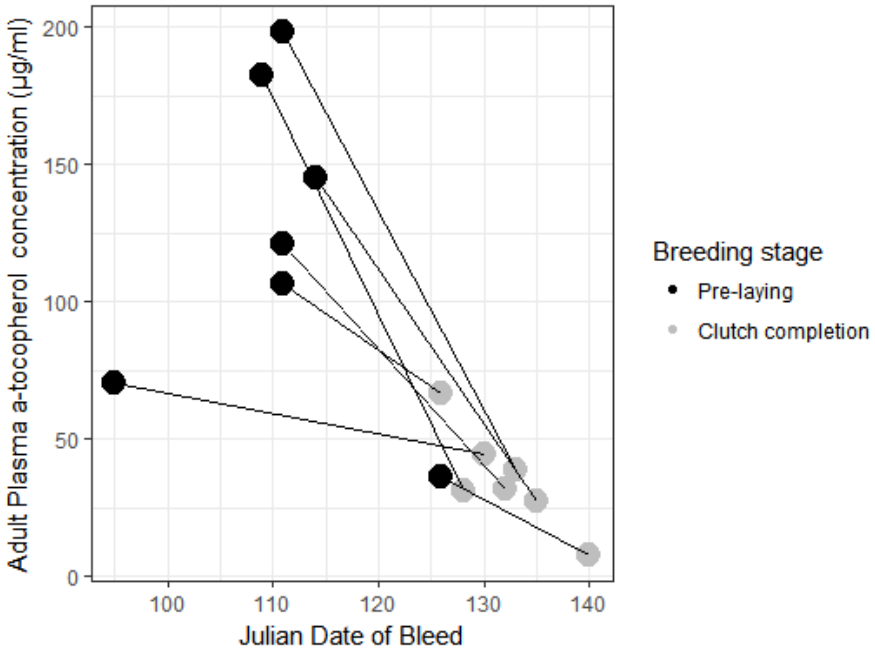

Figure 2.2. The change in α-tocopherol concentrations between Julian bleed date at pre-laying and clutch completion

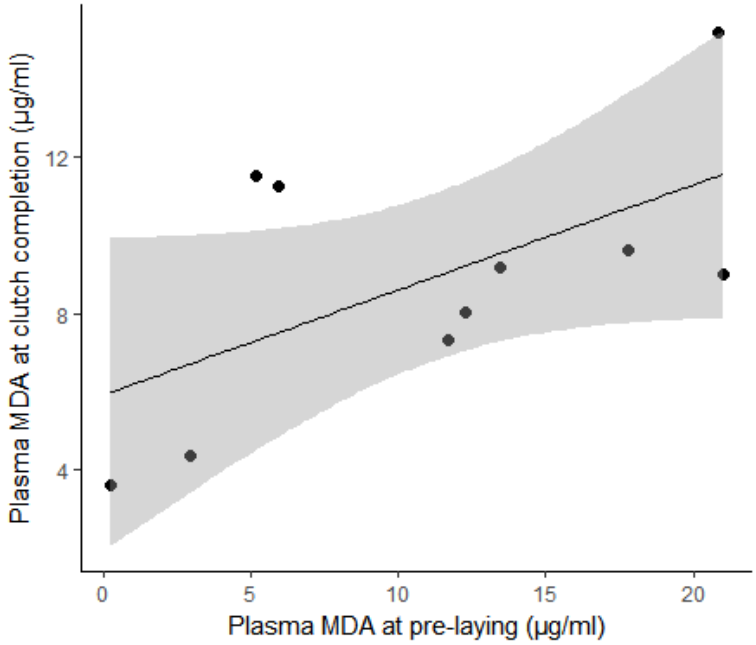

Figure 2.3. Relationship between plasma MDA concentration pre-laying and at clutch completion with 95% confidence intervals

In contrast, for all females, plasma α-tocopherol levels decreased between pre-laying and clutch completion (cross-sectional GLMM with site as random factor: stage, χ^2 = 12.17, d.f. = 5, P = <0.001; laying date, χ^2 = 5.29, d.f. = 6, P = 0.021; stage x laying date interaction, NS; plasma α-tocopherol at pre-laying: 122.78 ± 21.99 µg/ml; plasma α-tocopherol at clutch completion: 35.29 ± 6.78 µg/ml: GLMM for repeated measures with individual and site as random factors: stage, χ^2 = 11.87, d.f. = 5, P = <0.001; laying date χ^2 = 0.52, d.f. = 6, P = 0.47; stage x laying date interaction, NS; Figure 2.2). Using cross sectional data, there was no association between plasma levels of MDA and α-tocopherol either pre-laying (GLMM with site as a random factor: α-tocopherol, χ^2 = 1.66, d.f. = 4, P = 0.20) or at clutch completion (GLMM with site as a random factor: α-tocopherol, χ^2 = 0.40, d.f. = 4, P = 0.53).

2. Relationships between pre-laying oxidative state and egg production (2016 only)

Independently of laying date, females with lower plasma concentrations of MDA and higher plasma concentrations of α-tocopherol pre-laying produced significantly larger clutches (Table 2.1. and Figure 2.4 & 2.5). Eggs laid by females with higher plasma levels of MDA pre-laying had lower concentrations of MDA in yolk (Table 2.1). However, an increase in plasma MDA between pre-laying and clutch completion was marginally associated with higher yolk levels of MDA(GLMM with site as a random factor: absolute change in plasma MDA, $\chi^2 = 3.70$, d.f. = 4, P = 0.054; Figure 2.6). Females who laid later in the season and had higher pre-laying levels of α-tocopherol and/or MDA laid eggs with higher yolk concentrations of α-tocopherol, irrespective of clutch size (Table 2.1).

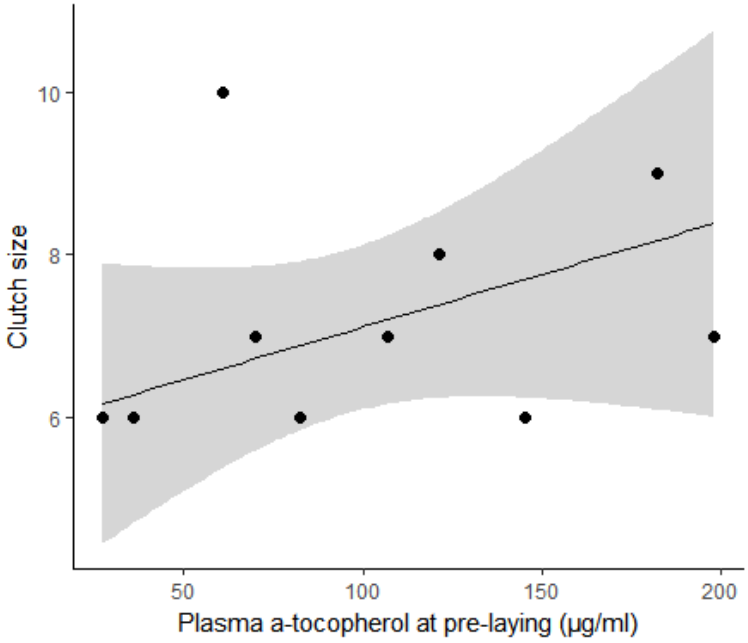

Figure 2.4. The predicted relationship between female plasma α-tocopherol concentration at pre-laying and clutch size with 95% confidence intervals

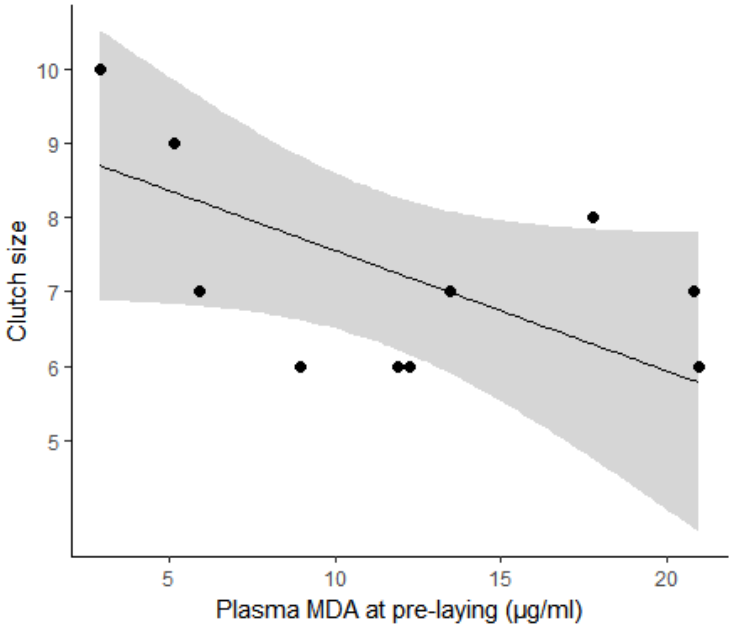

Figure 2.5. The predicted relationship between female plasma MDA concentration at pre-laying and clutch size with 95% confidence intervals

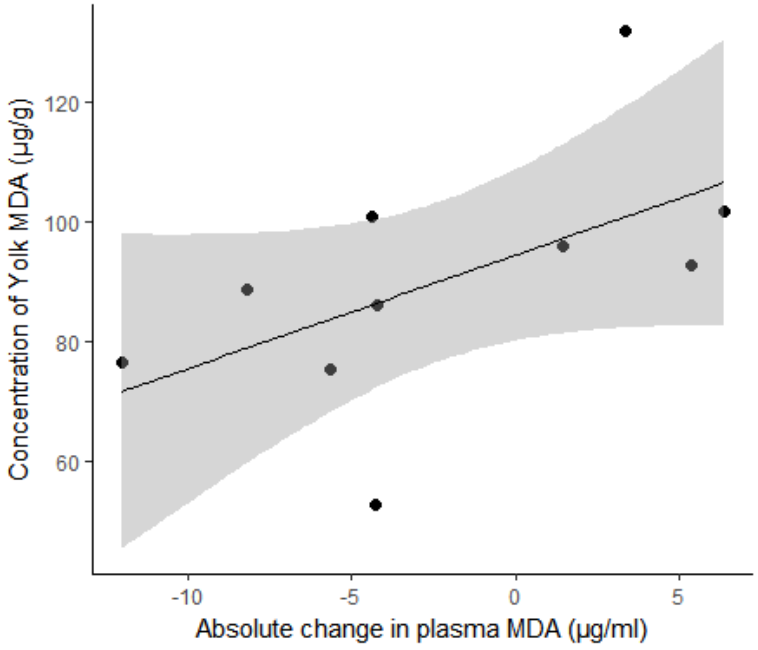

Figure 2.6. Relationship between the absolute change in plasma MDA and the concentration of yolk MDA with 95% confidence intervals

Table 2.1. Final model of the relationships between female oxidative state at pre-laying and egg production (2016 only).

Dependent variable	N (females)	Fixed Effects	Estimate ± SE	D.F.	χ^2	P
a) Clutch size	9	Pre-laying plasma MDA	-0.173 ± 0.06	5	7.88	0.005*
	9	Pre-laying plasma α-tocopherol	0.012 ± 0.007	5	3.97	0.046*
	9	Laying Date	-0.02± 0.07	6	0.12	0.75
b) Yolk MDA concentration	10	Pre-laying plasma MDA	-1.96 ± 0.71	4	6.64	0.01*
	10	Laying date	-0.22 ± 0.82	5	0.10	0.75
	10	Clutch size	-1.05 ± 5.42	6	0.06	0.80
c) Yolk MDA concentration	8	Clutch size	5.89 ± 3.77	4	2.74	0.10
	8	Laying date	0.69 ± 0.84	5	1.01	0.31
	8	Pre-laying plasma α-tocopherol	-0.05 ± 0.10	6	0.38	0.54
d) Yolk α-tocopherol concentration	8	Pre-laying plasma MDA * laying date	0.70 ± 0.45	7	4.79	0.029*
	8	Clutch size	-2.46 ± 13.42	7	0.09	0.77
e) Yolk α-tocopherol concentration	8	Pre-laying plasma α-tocopherol * laying date	0.08 ± 0.04	7	4.98	0.026*
	8	Clutch size	5.23 ± 8.35	7	0.21	0.65
f) Egg mass	10	Laying date	-0.01 ± 0.003	4	4.04	0.044*
	10	Pre-laying plasma MDA	-0.003 ± 0.00	5	0.68	0.41
	10	Clutch size	-0.02 ± 0.02	6	0.88	0.35
g) Egg mass	8	Laying date	-0.004 ± 0.00	4	1.95	0.16
	8	Pre-laying plasma α-tocopherol	-0.00 ± 0.00	5	2.23	0.14
	8	Clutch size	0.01 ± 0.01	6	0.56	0.45

3. Egg phenotypes (2010 and 2016)

There was no influence of year or laying date on yolk MDA (GLMM: year, χ^2 = 0.29, d.f. = 4, P = 0.59; laying date, χ^2 =0.30, d.f. = 5, P = 0.586; year x laying date interaction, NS), or egg mass (GLMM: year, χ^2 = 0.00, d.f. = 5, P = 0.97; laying date, χ^2 = 0.85, d.f. = 4, P = 0.36; year x laying date interaction, NS) However, levels of yolk α-tocopherol were significantly higher in 2010 (2010: 140.59 ± 11.20 μg/g; 2016: 94.64 ± 8.33 μg/g: GLMM: year, χ^2 = 10.02, d.f. = 4, P = 0.002; laying date, χ^2 = 0.38, d.f. = 5, P = 0.54; year x laying date interaction, NS). Clutches laid earlier in the season were larger in both years (GLMM: laying date, β = -0.11 ± 0.03, χ^2 = 9.84, d.f. = 4, P = 0.002; year χ^2 = 0.10, d.f. = 5, P = 0.76; year x laying date interaction, NS).

4. Relationships between yolk MDA and α-tocopherol, and offspring survival

Hatching success was marginally higher in 2010 compared to 2016, but the difference was not statistically significant (number of chicks hatched; 2010, 7.04 ± 0.36; 2016, 6.13 ± 0.33; t-test: t = 1.87, d.f. = 45, P = 0.068). However, there were no significant influences of laying date, yolk levels of MDA or α-tocopherol on hatching success (Table 2.2a.). Fledging success was significantly lower in 2016 than 2010 (number of chicks to fledge: 2010, 4.74 ± 0.63; 2016, 2.58 ± 0.59; t-test: t = 2.51, d.f. = 45, P = 0.016). However, while eggs laid earlier in the season had higher levels of α-tocopherol and greater fledging success, clutches that had higher yolk concentrations of MDA had significantly lower fledging success in both years (Table 2.2b. and Figure 2.7). All other fixed terms and two-way interactions were non-significant.

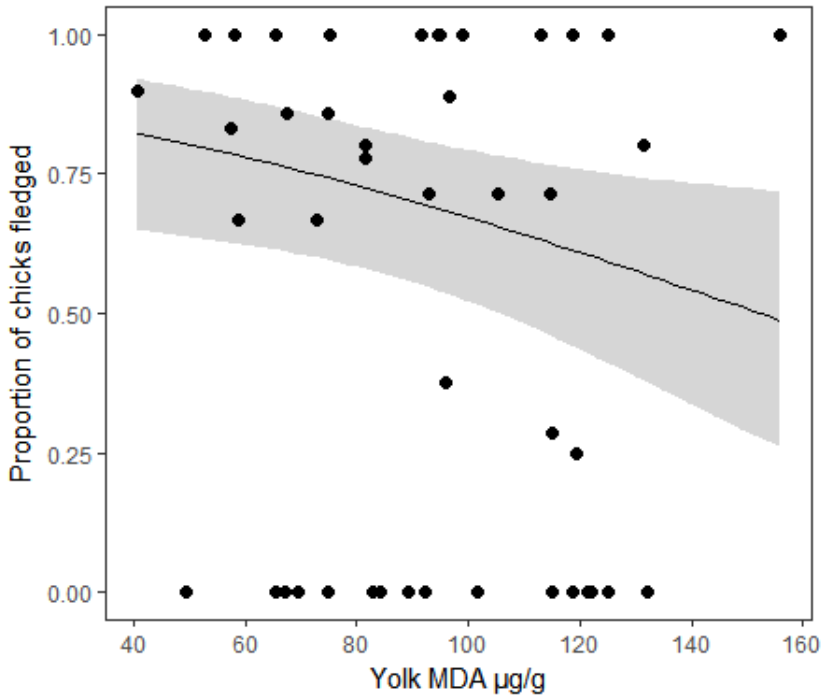

Figure 2.7. The predicted relationship between yolk MDA concentrations and fledging success with 95% confidence intervals

Table 2.2. Final model of the relationships between yolk MDA and α-tocopherol concentrations on hatching and fledging success

2a.Hatching success

Fixed effects	Factor level	Estimate ± SE	χ^2	D.F.	P
Year	2010	2.86 ± 0.47	4.76	3	0.029*
	2016	-0.96 ± 0.48			
Yolk MDA		-0.28 ± 0.19	2.05	4	0.15
Laying date		0.11 ± 0.28	0.18	5	0.68
Yolk α-Tocopherol		0.02 ± 0.29	2.12	6	0.96

2b. Fledging Success

Yolk α-tocopherol* laying date		1.31 ± 0.29	22.50	5	<0.001*
Yolk MDA		-0.39 ± 0.17	5.82	6	0.016*
Year	2010	1.02 ± 0.39	0.46	7	0.50
	2016	-0.28 ± 0.42			

Discussion

We investigated longitudinal changes in female oxidative state over the course of a reproductive attempt, and the relationships with offspring production and survival in wild blue tits. There was considerable variation in plasma levels of MDA pre-laying, although much of that variation had disappeared by the time of clutch completion. Such pre-laying plasma MDA levels were important because they predicted clutch size. Females with higher plasma levels of malondialdehyde (lipid peroxidation) prior to laying subsequently produced smaller clutches, suggesting that oxidative stress may constrain fecundity. In addition, we have, for the first time, shown that maternally-derived oxidative damage in the form of MDA is present in the yolk of freshly laid eggs. While levels of yolk MDA had no influence on hatching success, broods from clutches with higher levels of yolk MDA had lower survival to fledging.

We found no significant changes in female oxidative state from pre-laying through clutch completion and provisioning. However, there was large individual variation in pre-laying oxidative state and the subsequent direction of change in plasma MDA concentrations. When females were categorised as having either ‘high’ or ‘low’ levels of MDA pre-laying, it was revealed that individuals with relatively high levels of oxidative damage during the pre-laying period showed diminished levels of MDA at clutch completion. In contrast, females with relatively low levels of MDA pre-laying showed a marginal increase in MDA levels at clutch completion. Consequently, at clutch completion ‘high’ and ‘low’ category females had rather similar plasma MDA levels. Given the high metabolic cost of egg production (Heaney & Monaghan 1995; Monaghan et al. 1995; Monaghan & Nager 1997; Monaghan et al. 1998) it is perhaps surprising

that plasma levels of MDA did not increase in all females while they made a clutch of eggs. On the contrary, our data suggest that female oxidative state had converged towards a similar phenotype by the time that laying was complete. Such convergence in maternal plasma MDA levels at clutch completion was not simply due to maternal transfer of MDA from plasma into egg yolk, as we observed no correlation between the amount of depletion in females and the levels within the yolk. Indeed, an increase in plasma MDA between pre-laying and clutch completion was marginally significantly associated with higher yolk levels of MDA. This suggests that deposition of MDA into yolk is an unavoidable consequence of the increase in metabolic rate associated with egg production and massive catabolism of stored reserves (Monaghan & Nager 1997; Nilsson & Råberg 2001; Nager et al. 2000). It is possible that the oxidative status of eggs varied within the clutch, potentially due to laying order, and therefore the rate of passive transfer of MDA or antioxidants may vary depending on which egg was sampled. Therefore, further information on the oxidative status of the complete clutch could provide greater detail on maternal transfer of MDA with respect to laying order, and the potentially intricate relationship between maternal oxidative status and egg quality.

Due to the nature of studying physiological changes within subjects in the field, sampling events of adult birds were inevitably connected to the progression of the spring season. Therefore, any influence of temperature or dietary changes as a consequence of the changing season were unable to be disentangled from sampling events. Subsequently, any observed changes in physiology may, in full or in part, be due to natural physiological changes influenced by the season. Thus, to further validate this field study, aviary based studies would greatly help

to disentangle the influences of seasonal progression and reproduction on female reproductive physiology.

Our results show that females with higher oxidative damage at the pre-laying period produced smaller clutches. This negative association between female oxidative status pre-laying and her ability to invest in egg production could be an indication of an oxidative constraint on reproduction.Such a constraint has been observed elsewhere (Stier et al. 2012; Bertrand et al. 2006; Bergeron et al. 2011; Xu et al. 2014), and oxidative status has also been shown to determine both the timing and number of eggs laid in experimentally stressed canaries (Costantini et al. 2016). Furthermore, females with higher plasma levels of α-tocopherol laid larger clutches, supporting the idea that high vitamin E levels indicate a good nutritional status and therefore greater clutch capacity. However, it may also indicate the importance of lipid-soluble antioxidants within the vitamin E group that provide crucial protection against oxidative stress in females by breaking peroxidation reaction chains and neutralising ROS (Sies 2001). Whether levels of α-tocopherol in females at pre-laying are merely indicative of nutritional status or are actively accumulated to provide protection against oxidative stress at this crucial stage of reproduction is yet to be investigated.

This study provides the first empirical evidence of oxidative damage being passed to eggs with detrimental effects. While there has been a wealth of research into the benefits of maternal effects via the egg, there has been little investigation into whether harmful substances are also passed to eggs obligatorily with negative implications.The adaptive benefits of maternally derived immunoglobulins and hormones have been well studied (Marshall

&Uller 2007; Groothuis & Schwabl 2008) with the consensus that these phenotypic traits are of great importance for success via adaptive plasticity. In particular they help to equip offspring for the ecological situation into which they are born (Räsänen & Kruuk 2007; Groothuis et al. 2005). However, studies to date have largely focussed on hormone-mediated and postnatal maternal effects with little focus on the functional mechanisms behind these traits (Groothuis & Schwabl 2008). The present study offers the first evidence of transgenerational impacts of oxidative stress during egg production. (Blount et al. 2000). While previous studies have provided evidence of products of aldehydic lipid peroxidation, such as MDA, being passed into egg yolk (Mourente et al. 1999; Mohiti-Asli et al. 2008), they have not assessed the impacts on offspring. Furthermore, lipid-rich yolk tissue is highly susceptible to peroxidation (Hulbert et al. 2007; Wakefield et al. 2008; Surai et al. 1996) and elevated metabolic rates during embryogenesis can cause high levels of oxidative stress within developing eggs (Blount et al. 2000; Hulbert et al. 2007; Ayala et al. 2014). Thus, it is important to mention here that although there is the possibility that MDA may be internally derived within yolk, and also some evidence for the oxidative degeneration of yolk nutrients caused by storage over time (Grune et al. 2001), eggs used in this study were fresh (within 2 days of the onset of incubation) and immediately frozen at -80°C for analysis within 6 months, minimizing further peroxidation that could jeopardize results. It must be acknowledged however, that it is possible that embryo development within eggs started within two days of the onset of incubation. Thus, it is plausible that not all of the MDA detected in eggs was of maternal origin, as the process of oxidising PUFAs for embryo development may have begun before egg collection. Therefore, we cannot be wholly confident that the MDA which we

measured in eggs was representative of maternally-derived oxidative damage levels. However, for the purposes of this study we will consider that MDA detected within eggs was of maternal origin while acknowledging that the origins of this MDA requires further study within more easily controlled aviary conditions. It must also be acknowledged that the measurement of a single egg is not representative of the whole clutch and future studies would benefit from whole clutch analysis to assess variation in egg phenotype within clutches. Due to the small sample sizes and the longitudinal aspect of this study, this was not possible here but should be considered in further studies.

All females showed a reduction in levels of plasma vitamin E from pre-laying to clutch completion; an anticipated result likely caused by maternal investment of essential antioxidants in egg yolk (Royle et al. 2001; Perrins 1996; P.F.Surai 2000).In contrast to some previous studies (Bize et al. 2008; McGraw et al. 2005) we did not find that the levels of antioxidants in eggs significantly affected hatching success. Interestingly, however, our results show that levels of lipid peroxidation in yolk predicted fledging success. We have already seen that females with higher levels of pre-laying MDA produced smaller clutches, so the influence of oxidative stress pre-laying appears to manifest in a constraint both on egg number and egg quality. It is possible that this result is the product of sampling design, whereby the last laid egg in a larger clutch is of lower quality than the last laid egg in a smaller clutch and subsequently, proportionately fewer chicks fledge in a larger clutch. However, it has been suggested that females are unlikely to produce eggs of such poor quality that they will be unviable (Monaghan et al. 1995; Monaghan et al. 1998; Monaghan & Nager 1997; Visser & Lessells 2001).To remove this limitation in study design, future studies could consider either standardising which egg is removed (eg. egg

number 5) or using a species with a determinate number of eggs. Our findings suggest that the likelihood that eggs will give rise to surviving offspring is a complex function of both beneficial (e.g. vitamin E) and harmful (MDA) compounds transferred from mother to young.

There may be some coevolution of traits within mothers and offspring where selection is acting both on maternal and offspring fitness and within ecological timescales (Räsänen & Kruuk 2007; Crespi & Semeniuk 2004; Wolf, Jason B 2009). Such a parent-offspring conflict could result in a non-significant effect of oxidative stress on hatching success because of a coevolved threshold where adults achieve the maximal clutch size with no direct effect on egg viability. We found no relationship between clutch size and the amount of lipid peroxidation within yolk, which suggests that individuals produce eggs to a maximal investment threshold. Given that egg quality is largely determined by environmental conditions experienced by the parents, such as the availability of specific nutrients (Perrins 1996; Blount et al. 2000; P.F.Surai 2000; Surai et al. 2016), the oxidative status of the yolk is determined by maternal influences. Subsequently, the oxidative status of the yolk directly determines the quality of tissues within the chick (Surai 2000; Surai et al. 2016; Parolini et al. 2017), and this is primarily tested after hatching (Mohiti-Asli et al. 2008). Poorly protected chicks may be seriously harmed when they are subjected to high levels of atmospheric oxygen at hatching which can cause a burst of oxidative stress (Al-Gubory et al. 2010; Agarwal et al. 2005). Thus, the oxidative status of offspring is more likely to determine chick survival once they have hatched, and may be largely dependent on environmental conditions. Indeed, in red-winged black birds a supplementary increase in dietary antioxidants allowed nestlings to increase growth rate with no observable costs of doing so, suggesting oxidative

stress is a limiting factor in offspring growth and survival (Hall et al. 2010). Resistance to oxidative stress in great tits has been shown to have low heritability and high environmental dependence in cross fostering studies (Losdat et al. 2014), suggesting that the impact of acquiring maternally derived oxidative damage on fledging success may be largely manipulated by the common environment. However, as previously mentioned, females with higher levels of oxidative damage at clutch completion had greater fledging success. This implies firstly that there is a cost associated with better quality offspring (Alonso-Álvarez et al. 2010a), and secondly that maternal investment in egg quality is equally as important as environmental influences on offspring survival.

There remains intense discussion as to the role of oxidative stress as a constraint, or a cost of reproduction (Isaksson et al. 2011; Metcalfe & Monaghan 2013; Selman et al. 2012; Speakman & Garratt 2014; Blount et al. 2016), Our findings add partial support to the 'oxidative shielding' hypothesis (Blount et al. 2016), which suggests that mothers should pre-emptively reduce levels of oxidative damage on transition to reproduction in order to protect themselves, and in particular their physiologically dependent offspring, from harm caused by the inevitable increase in oxidative damage which ensues through the expenditure of reproductive effort. In contradiction to this hypothesis we did not find a reduction in oxidative damage levels during egg production, compared to pre-laying or during chick provisioning. However, females may have been investing in eggs to their individual optima, which may mask or obscure any such 'shielding' effect in the absence of an experimental manipulation of egg number (Blount et al. 2016). Importantly, however, we found that higher levels of MDA in yolk were associated with lower fledging success, so clearly there must be a premium on mothers to minimise their own

MDA levels during egg production in order to protect their offspring from transgenerational damage. Across taxa there is now mounting evidence that females do indeed show decreased levels of oxidative damage during sensitive stages of reproduction when offspring may be susceptible to harm, e.g. lactation in mammals (Bouwstra et al. 2008; Garratt et al. 2011). Furthermore, in banded mongooses *Mungosmungo* plasma levels of MDA in pregnant mothers predicted offspring survival to emergence from the den at one month of age (Vitikainen et al. 2016).

In conclusion, our results support the idea that oxidative stress may constrain reproduction, while maternal transfer of oxidative damage to offspring via the egg is an important and hitherto unexplored component of the cost of reproduction that is borne in part by offspring. Future advances in our understanding of the costs of reproduction would therefore benefit from a greater appreciation of the potential for transgenerational impacts of the mechanisms underpinning life history trade-offs.

Chapter 3

The consequences of vitamin E provisioning on the susceptibility of lipids to peroxidation in egg yolk of the blue tit *Cyanistes caeruleus*

Miranda A.R. Walter[1],Kate E. Plummer[2]& Jonathan D. Blount[1]

[1]*Centre for Ecology and Conservation, College of Life and Environmental Sciences, University of Exeter, Penryn Campus, Cornwall, TR10 9FE, UK*

[2]*British Trust for Ornithology, Daphne du Maurier Building, University of Exeter, Penryn Campus, Penryn, Cornwall, TR10 9FE, UK*

Authors for correspondence: marw201@exeter.ac.uk; j.d.blount@exeter.ac.uk

Abstract

The balance between oxidative stress and antioxidant control is considered to be a crucial mechanism underpinning life history trade-offs and determining reproductive decisions. However, the relationship between oxidative stress and antioxidant activity remains unclear. It was recently proposed that oxidative stress may be transmitted to offspring via maternal effects in eggs, presenting a secondary oxidative cost of reproduction born by the offspring. Here we present data suggesting that a product of lipid peroxidation (malondialdehyde) is indeed passed to eggs, leaving developing embryos both susceptible to further destruction from aldehyde reactivity and comprising pre-damaged lipids. However, we find that pre-embryonic yolks with higher concentrations of vitamin E are less susceptible to further lipid peroxidation from an external stimulus. We suggest that a crucial functional role of vitamin E within egg yolk is to prevent lipid peroxidation *in-vivo* during incubation and early embryogenesis, prior to uptake of antioxidants in the latter stages of offspring development.

Introduction

It is broadly accepted that optimal investment in life history traits is constrained by trade-offs that prevent their simultaneous increase (Stearns 1992). While the proximate mechanisms are still much debated (Isaksson et al. 2011; Selman et al. 2012; Metcalfe & Monaghan 2013; Speakman & Garratt 2014), oxidative stress has been proposed as a functional mechanism that may underpin life history trade-offs. Metabolically demanding processes such as reproduction can lead to a rise in the production of reactive oxygen species (ROS) via oxidation-reduction reactions during ATP synthesis in the mitochondria (Dowling & Simmons 2009; Barja 2007; Costantini 2008; Monaghan et al. 2009). These ROS can overwhelm the antioxidant defences and cause oxidative stress. A consensus on the role of oxidative stress as a cost of reproduction remains elusive (Isaksson et al. 2011; Selman et al. 2012; Stier et al. 2012; Speakman & Garratt 2014; Metcalfe & Monaghan 2013), and further examination of the relationship between parental and offspring oxidative stress is needed to advance understanding in this field. In particular, the fitness impacts on developing offspring of incurring oxidative stress derived from parental reproductive effort is still largely unknown (Blount et al. 2016).

Maternal effects comprise a class of phenotypic effects whereby maternal phenotype directly effects the phenotype of her offspring and is unrelated to the offspring's genotype (Bernardo 1996). Eggs provide an ideal study system for maternal effects research, being a self-contained package with all the nutrients required for the developing embryo (Bernardo 1996). However, avian egg production is an energetically expensive process (Monaghan & Nager 1997) so oxidative stress incurred during egg production could be deleterious for the

viability of the egg if yolk functions such as antioxidant activity are impaired (Blount et al. 2000). Indeed, products of aldehydic lipid peroxidation, such as MDA, are incorporated into egg yolk (Mourente et al. 1999; Mohiti-Asli et al. 2008) and may cause further oxidative degeneration of yolk *in vivo*(Grune et al. 2001). Furthermore, lipid-rich yolk tissue, with high levels of poly-unsaturated fatty acids (PUFAs), is extremely susceptible to peroxidation (Hulbert et al. 2007; Wakefield et al. 2008; Surai et al. 1996). During embryogenesis, elevated metabolic rates and catabolism of PUFAs in yolk can cause high levels of oxidative stress (Blount et al. 2000; Hulbert et al. 2007; Ayala et al. 2014) which may present an additional oxidative burden on developing offspring, although this outcome is yet to be tested. Antioxidants and MDA in yolk may therefore be reactive from the moment of laying, even prior to embryogenesis and chick development.

Maternal provisioning of antioxidants into egg yolk is thought to protect embryos from oxidative stress during development (Blount et al. 2000; Grune et al. 2001; Surai & Speake 1998; Surai 2002; Surai et al. 1996; Surai et al. 2016). Indeed, much attention has been given to study of the transmission of antioxidants from parents to offspring in avian systems, including the adaptive advantages that can be provided in eggs via such maternal effects (Blount et al. 2000; Surai et al. 1999b). Experimental diet manipulation studies have shown that provisioning mothers with enhanced antioxidant diets can result in higher levels of these antioxidant components in yolk and developing chick tissues, resulting in subsequently lower offspring susceptibility to oxidative stress (Surai 2000; Surai et al. 2016; Surai et al. 1999b; Lin et al. 2005; Mohiti-Asli et al. 2008). In particular, vitamin E and carotenoids have been the focus of inherited antioxidant protection because of their roles in neutralising ROS activity.

Vitamin E serves as a major chain-breaking antioxidant, influential in quenching oxyradicals and interrupting sequences of oxidation (Blount et al. 2000; Surai 2000). Carotenoids are multifunctional biologically active pigments that are crucial to the composition of egg yolk because they act as an antioxidant, reducing lipid peroxidation within the developing embryo (Al-Gubory et al. 2010; Blount et al. 2000; Blount et al. 2002; Finkel & Holbrook 2000; Surai 2002, but see Hartley & Kennedy 2004; Costantini & Møller 2008). There is, however, still much uncertainty as to the functional role of carotenoids as an antioxidant because they constitute a very small proportion of antioxidant capacity in birds (Costantini & Møller 2008) and are themselves susceptibility to oxidative attack, relying on other antioxidants, namely vitamins C and E, for protection (Hartley & Kennedy 2004). The maternal provisioning of antioxidants such as vitamin E and debatably carotenoids into eggs may be essential in influencing offspring susceptibility to oxidative stress (Catoni et al. 2008; Surai 2002; Surai et al. 2016). Importantly, both vitamin E and carotenoids are diet-derived, so maternal investment of these antioxidants into egg yolk may be limited (Surai & Speake 1998; Isaksson 2009), potentially generating a parent-offspring trade-off for antioxidant protection.

However, while antioxidant provisioning in eggs seems likely to be important for offspring fitness, little is known about the oxidative conditions that may determine such provisioning in wild populations of birds. Indeed, while there is much evidence of antioxidant transmission from mother to egg, data on the transmission and impact of maternally derived oxidative stress is scarce (Blount et al. 2016). There is evidence of maternal transmission of detrimental compounds such as arsenic (Kubota et al. 2002), lead (Chatelain et al. 2016) and other heavy metals (Mora 2003) in bird eggs, suggesting that mothers do

transmit harmful compounds to eggs, presumable involuntarily as they can't avoid doing so. It is therefore possible that mothers could pass on naturally occurring detrimental components if the process is unavoidable. Studies on wild, free living systems would provide important information on the ecological context in which the functional mechanisms that underpin maternal provisioning have evolved. These should include natural influences, such as dietary antioxidant availability, which may determine the level of maternal investment and thus, the susceptibility of offspring to peroxidation during embryogenesis.

Here, we used wild populations of blue tits *Cyanistes caeruleus* to examine yolk composition, including a marker of lipid peroxidation (melanodialdehyde – MDA) and antioxidants (α-tocopherol and total carotenoids). We tested the response of these components to an oxidative stimulant (iron; FeSO4) *in vitro*, and observed the magnitude of yolk susceptibility to lipid peroxidation dependent on levels of antioxidants within the yolk.We hypothesised that low concentrations of antioxidants (α-tocopherol and carotenoids) would be associated with high concentrations of MDA in freshly laid egg yolk. We further hypothesised that introduction of FeSO4 to yolk tissue *in vitro* would induce an increase in yolk MDA. Finally, we hypothesised that this experimental increase in yolk MDA would be greatest in eggs that had the lowest yolk concentrations of antioxidants.

Methods

Study system

The study was carried out during spring in 2010 and 2015 in Cornwall, UK, at five deciduous woodland sites (Grid refs: SW7837, SW7737, SW7537, SW8240) ranging from 9.5 to 15 hectares in area. The predominant tree species were oak (*Quercus*spp.), beech (*Fagussylvatica*), sweet chestnut (*Castaneasativa*) and sycamore (*Acer pseudoplatanus*). Nest boxes were distributed along transects at 25 metre intervals. Nest boxes were monitored for nest building activity from mid-March; upon the initiation of nesting boxes were checked every other day, and once egg laying commenced boxes were examined daily until clutch completion. Newly laid eggs were weighed on a portable balance to 0.01g (ProScale 111, E-scales, Chester, UK), measured using a digital metal calliper (Stainless Steel Electronic Digital VernierCaliper 0-150mm, Simply Bearings Ltd. UK), and marked using a fine point marker pen to indicate laying order. Clutches were deemed to be complete when two days had elapsed with no further eggs being laid, and incubation had commenced. At this point the last-laid egg was removed and stored at -80°c for biochemical analysis. Mean Julian laying dates reported as mean day ± SE (2010, 121 ± 1.05; 2015, 115 ± 0.88).

Biochemical Analyses

Malondialdehyde (MDA) and addition of FeSO4

Yolk MDA was determined using High-Performance Liquid Chromatography (HPLC) as described previously (Plummer et al. 2013). Yolk (40-50 mg) was

placed in phosphate buffered saline (PBS) at 5% w/vand then homogenised for 30 sec using a T18 Basic Ultra-Turrax® homogeniser (IKA® England LTD). To test the susceptibility of yolk to FeSO4 induced oxidative damage, 200 µl of yolk homogenate was transferred to each of 3 reaction tubes. 10 µl of 2.1M FeSO4 solution was added to the first tube to yield a final concentration of 0.1mM FeSO4 (+FeSO4), and 10 µl of demineralised milli Q water (control) was added to the second sample as a control. The third sample as left untreated. Samples were vortexed for 5 sec then placed on a hot plate at 37 C which was placed on a shaker at 100rpm for 20 min. Samples were then transferred to ice for 5 min. A 20µl aliquot of homogenate from each treatment was then transferred to reaction tubes pre-loaded with 20µl butylatedhydroxytoluene (BHT). Thiobarbituric Acid (TBA) (40µl) and phosphoric acid (160µl) were added, and the mixture was vortexed for 5 sec before being placed in a dry heat bath at 100 C for 1h. Samples were then cooled on ice, 100µl of butanol was added and samples were vortexed for 20 sec, then centrifuged at 4 C, 12,000 x g for 3 min. An aliquot (60µl) of the upper phase, containing MDA-TBA adducts, was drawn off and placed in an HPLC vial. A 20µl aliquot was injected into an HPLC system (Dionex Corporation, California, USA) fitted with a Hewlett-Packard Hypersil 5µ ODS 100 x 4.6 mm column and a 5µ ODS guard column (Thermo Fisher Scientific Inc. Massachusetts, USA), maintained at 37C. The mobile phase was methanol-buffer (40:60 v/v) at a flow rate of 1ml min^{-1}, the buffer being 50mM potassium monobasic phosphate adjusted to pH 6.8 using 5M potassium hydroxide. Fluorescence detection (Dionex RF2000) was performed at 515 nm (excitation) and 553 nm (emission). Yolk MDA concentrations were calibrated using an external standard of 1,1,3,3-tetraethoxypropane (TEP) serially diluted with 40% ethanol.

α-Tocopherol and Carotenoids

For extraction of antioxidants, egg yolk (40-50mg) was vortexed in 0.7mL 5% NaCl for 5 sec and then homogenised with 1mL EtOH for 20 sec. 1.5mL hexane was added and further homogenised for 10 sec. The sample was centrifuged at 8,000 × g for 4 min, and the hexane phase containing the antioxidants drawn off. A further 1.5mL hexane was added for a second extraction and both hexane extracts were then combined.

Total carotenoid concentrations were determined by measuring absorbance at 450nm using a Nicolet Evolution 500 spectrophotometer (Thermo Electron Corp., Hemel Hemstead, UK), and the extinction coefficient of lutein in hexane (2589; Craft &Soares 1992). Yolk α-tocopherol concentration was determined by HPLC as described previously (Plummer et al. 2013). A 500µl aliquot of hexane extract was dried under nitrogen gas and then re-dissolved in 150µl dichloromethane and 150µl methanol. Samples (20µl) were injected into a Dionex HPLC system (Dionex Corporation, California, USA). Separation utilized a 3-µ C_{18} reverse-phase column (15 cm × 4·6 mm) (Spherisorb S30DS2; Phase separations, Clwyd, UK), with a mobile phase of methanol (HPLC grade)/water (97 : 3 v/v) at a flow rate of 1.1 mL min^{-1}. Fluorescence detection (Dionex RF2000) was performed at 295 nm (excitation) and 330 nm (emission). The α-tocopherol peak was identified and quantified by comparison with a standard solution of α-tocopherol (T3251 Sigma-Aldrich) in methanol. Total carotenoid and α-tocopherol concentrations are reported as µg g^{-1} yolk.

Statistical analysis

All statistical analyses were performed using R studio version 3.3.3 (Copyright© 2017 The R Foundation for Statistical Computing). Data were analysed using analysis of variance (ANOVA) and general linear mixed effect models (GLMMs) using the lme4 package with backwards stepwise deletion of non-significant terms based on AIC, starting with the interaction terms, where alpha was set at 0.05.

Analyses of the difference in yolk MDA concentrations in relation to FeSO4 treatment utilised ANOVA with treatment as a factor (untreated, control, +FeSO4), and including laying date as a covariate. A repeated measures ANOVA was then used to analyse the difference within individual yolks controlling for initial untreated yolk MDA. Repeated measures ANOVA post hoc analysis used Bonferroni adjusted Tukey's test. The absolute change in yolk MDA was tested using a GLMM, with yolk α-tocopherol and carotenoids by laying date as interacting terms to control for seasonal variation in antioxidants. Site was included as the random term. Control yolk MDA was also included as a covariate. All main effects and any significant two-way interactions are reported. Values are reported as means ± SE.

Results

1. Relationships between year, laying date, clutch size and yolk oxidative state

There was no significant difference in concentrations of yolk MDA between 2010 and 2015, but concentrations of yolk MDA were lower when clutch sizes

were larger earlier in the season, resulting in an overall significantly negative influence of clutch size by laying date on yolk MDA across both years (2010 = 94 ± 6.01 µg/g, 2015 = 111.40 ± 4.66 µg/g: GLMM: laying date x clutch size χ^2 = 9.33, d.f. = 6, P = 0.002; year χ^2 = 1.82, d.f. = 7, P = 0.18). Mean concentrations of α-tocopherol did not significantly differ between years. Similarly, there was no influence of laying date or clutch size on concentrations of yolk α-tocopherol (2010 = 140.59 ± 11.20µg/g, 2016 = 190.84 ± 30.83µg/g: GLMM: year χ^2 = 1.20, d.f. = 4, P = 0.27; clutch size χ^2 = 0.46, d.f. = 5, P = 0.50; laying date χ^2 = 0.04, d.f. = 6, P = 0.85). Concentrations of yolk carotenoids were significantly higher in 2015 compared to 2010, and this was not influenced by clutch size or laying date (yolk carotenoids 2010 = 31.99 ± 2.50, 2016 = 47.98 ± 2.49µg/g; GLMM: year χ^2 = 10.12, d.f. = 4, P = 0.002; laying date χ^2 = 1.42, d.f. = 5, P = 0.23; clutch size χ^2 = 0.03, d.f. = 6, P = 0.86).

2. Relationships between yolk MDA, α-tocopherol and carotenoids

Levels of MDA were negatively predicted by levels of α-tocopherol in 2010, but there was no significant relationship in 2015 (Figure 3.1.). All other fixed effects and interactions between α-tocopherol or carotenoids and clutch size, laying date and year were non-significant (Table 3.1). Levels of α-tocopherol and carotenoids were not significantly correlated (GLMM: α-tocopherol χ^2 = 0.39, d.f. = 5, P = 0.53).

Table 3.1. The relationship between α-tocopherol and carotenoids on MDA (DV) in yolk between years

Fixed Effect	Factor Level	Estimate ± SE	Df	χ^2	P
Year * α-tocopherol	2010 2016	0.35 ± 0.12	6	8.83	0.0029*
Laying date		0.36 ± 0.70	7	0.32	0.57
Clutch size		1.44 ± 2.25	8	0.44	0.51
Yolk carotenoids		0.07 ± 0.23	9	0.09	0.76

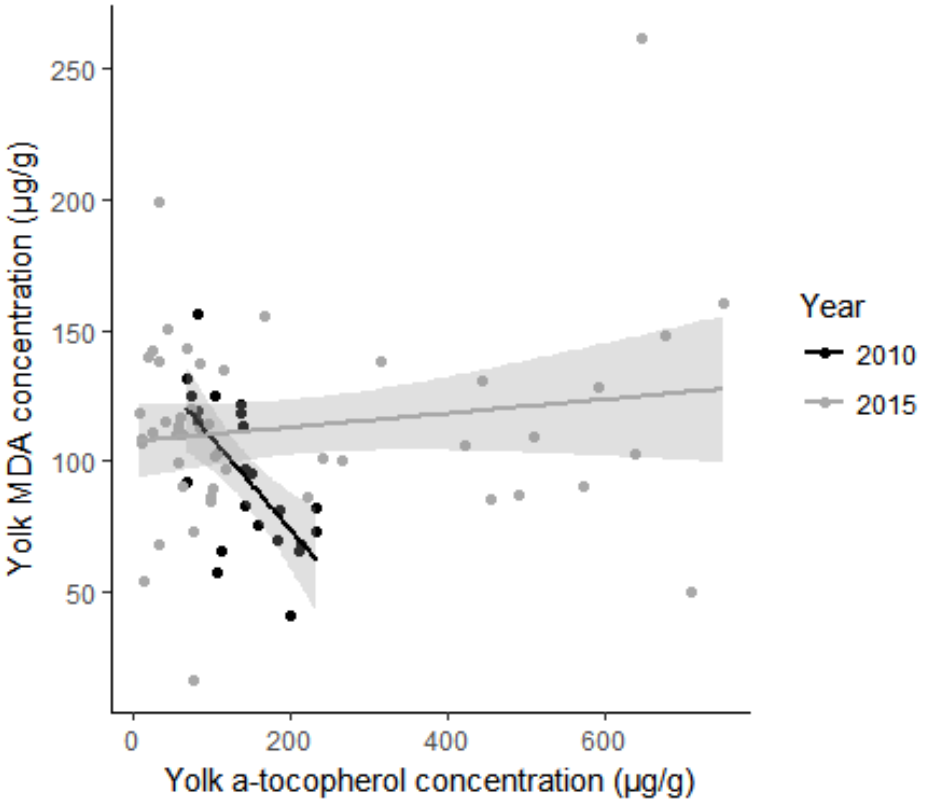

Figure3.1. The relationship between yolk MDA and α-tocopherol concentrations in 2010 and 2015 with 95% confidence intervals

3. Effects of yolk antioxidants on yolk susceptibility to lipid peroxidation

As expected, addition of iron (FeSO4) to yolk caused a significant increase in concentrations of MDA, while addition of water in yolk as a control had no

significant influence on MDA concentrations. These differences were apparent using one-way ANOVA (F = 346.8, d.f. = 2, P = <0.001; Tukey tests: FeSO4 versus untreated, P = <0.001, FeSO4 versus control, P = <0.001, untreated versus control P = 0.69; Figure 3.2.).

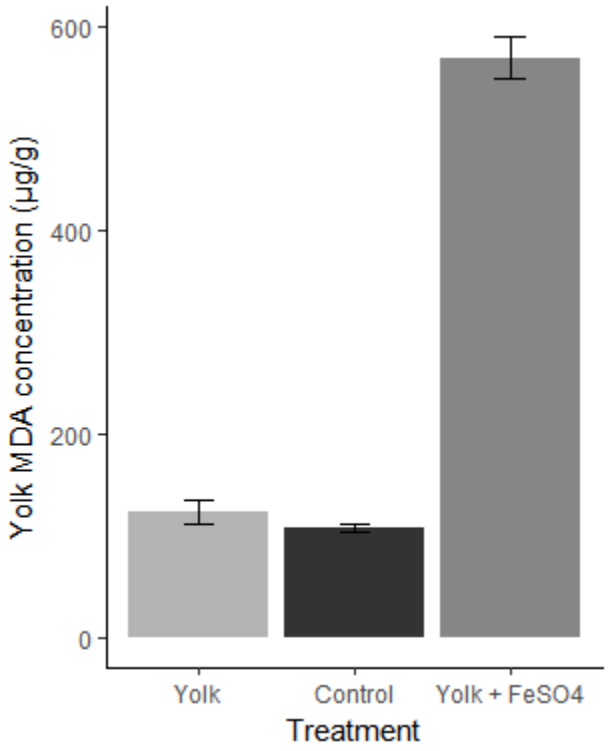

Figure 3.2. Yolk MDA concentrations in each treatment group with standard error. Yolk = untreated yolk, Control = yolk + water, Yolk + FeSO4 = yolk + FeSO4 exposure

As predicted, the increase in MDA concentrations after treatment with FeSO4 was greater in yolks that contained lower concentrations of α-tocopherol (Figure 3.3.). In contrast, concentrations of total carotenoids did not significantly affect the change in MDA following addition of FeSO4 (Table 3.2.). Laying date and the interaction between laying date with levels of carotenoids and α-tocopherol had no influence on the change in MDA after FeSO4 treatment (Table 3.2.).

Table 3.2. The influence of antioxidants on the susceptibility of yolk to lipid peroxidation. All main effects and significant interactions from GLMM minimum adequate model reported.

Fixed Effects	Estimate ± SE	D.F.	χ^2	P
α-tocopherol	-0.18 ± 0.09	4	4.22	0.040*
Carotenoids	0.81 ± 1.14	5	0.53	0.47
Laying date	-2.09 ± 2.62	6	0.68	0.41
MDA control	0.03 ± 0.59	7	0.002	0.96

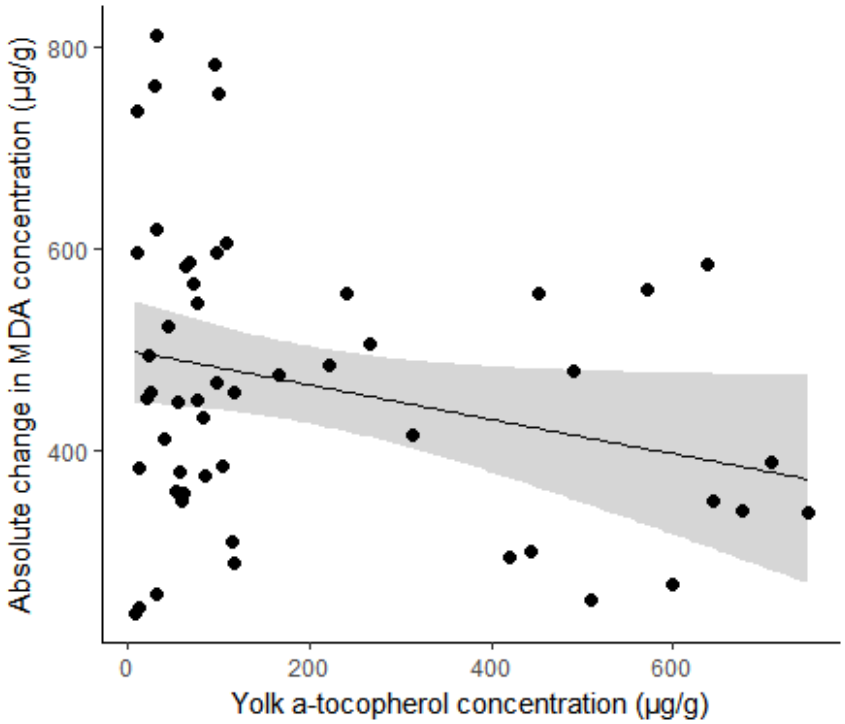

Figure 3.3. The predicted relationship between yolk concentrations of α-tocopherol and the absolute change in yolk concentrations of MDA after treatment with FeSO4 with 95% confidence intervals

Discussion

Using blue tit eggs, we examined the relationships between yolk concentrations of antioxidants and lipid peroxidation, including the susceptibility of yolk to increases in oxidative damage *in vitro* dependent on natural antioxidant levels. Concentrations of carotenoids did not significantly influence yolk susceptibility to lipid peroxidation. However, yolk with lower levels of α-tocopherol was more susceptible to lipid peroxidation from a pro-oxidant challenge, suggesting that maternal provisioning of eggs with vitamin E may serve to protect yolk against oxidative stress during embryogenesis. Furthermore, since vitamin E is diet-derived, it implies that the availability of these molecules to mothers may determine the level of oxidative protection that they can afford to their eggs, potentially causing a reproductive trade-off between adult and offspring antioxidant protection. Interestingly, yolk concentrations of MDA and α-tocopherol did not differ significantly between years. However, while levels of yolk α-tocopherol and MDA were negatively correlated in 2010, no such relationship was observed in 2015. The absence of a visible relationship may be due to some individuals having much higher α-tocopherol concentrations without correspondingly low MDA in 2015, which appears to mask the associations at lower concentrations. This suggests that seasonal differences between years may influence the availability of dietary antioxidants, thus influencing the association between yolk antioxidant provisioning and oxidative stress in reproductive investment.

To the best of our knowledge, data from our study are the first to demonstrate oxidatively damaged lipids are passed to offspring, presenting a potential oxidative cost of reproduction which is thus far unexplored. The transmission of

female derived MDA to yolk presents a two-fold oxidative challenge to offspring. It not only suggests that some yolk lipids are already degraded, but also that yolk is at further risk of damage during embryogenesis due to the reactivity of MDA. MDA is a product of polyunsaturated fatty acid peroxidation and is considered the most mutagenic of the aldehydes produced by lipid peroxidation (Ayala et al. 2014). It is a reactive mutagenic, able to move across membranes and cytoplasm away from its site of generation to modify protein and DNA function (Del Rio et al. 2005; Ayala et al. 2014; Davies 2000). The majority of yolk uptake of antioxidants by the embryo occurs in the late stages of embryogenesis and continues into the first few days after hatching (Surai et al. 1996; Surai 1999). This suggests that vitamin E is serving to protect yolk against damage accrual throughout the period of incubation and embryogenesis when the developing offspring is not utilising the yolk. To the best of our knowledge, this concept has not been directly investigated, but it would be interesting to conduct a comparative analysis on incubation rates and levels of yolk antioxidants across bird species to further understand this relationship. The oxidative cost of receiving parent-derived MDA and vitamin E via yolk could, therefore, have a significant impact on offspring fitness if it influences their oxidative status even before hatching. However, this study was unable to distinguish between maternally derived oxidative damage and that from other sources, such as *in-vivo* generation within the egg, and therefore these data cannot provide evidence wholly of maternal transfer of MDA. Further studies are required to disentangle the sources of MDA within eggs and build on the preliminary data gathered in this study.

Our results suggest that vitamin E may function to protect yolk lipids against accrual of further oxidative damage even before the onset of embryogenesis,

and yolk susceptibility to oxidative damage during embryogenesis is likely dependent on the presence of vitamin E. α-Tocopherol, the isomer of vitamin E measured in this study, is an important lipid-soluble antioxidant and provides crucial protection against oxidative stress by breaking peroxidation reaction chains and neutralising ROS (Sies 2001). Of the eight lipid-soluble tocopherols, α-tocopherol is the most ubiquitous in nature (Burton 1994) and is also the main isomer of vitamin E found in blue tit eggs. Supplementation of adult hens with vitamin E has been shown to increase levels of this compound in embryos and chicks, significantly decreasing susceptibility to lipid peroxidation compared with offspring produced by non-supplemented mothers (Surai et al. 1999a; Surai 2000). Similarly, an imbalance in the concomitant concentrations of PUFAs and vitamin E in favour of PUFAs in Leghorn laying hens resulted in greater peroxidation due to insufficient vitamin E to protect against ROS attack (Grune et al. 2001). Our data support these findings and provide new evidence that eggs containing more vitamin E have superior protection against threats of peroxidation in pre-embryonic yolk. Indeed, it is possible that the level of antioxidant provisioning in yolk is determined by the extent of oxidative damage incurred by the female and is in fact primarily a mitigation mechanism.

Given that freshly laid eggs provide a snapshot of the female physiological state at the point of laying, the presence of MDA in yolk implies that females incur lipid peroxidation during egg formation. The products of such oxidative damage, ie. MDA, are likely to be passively transferred into yolk, similar to that observed in the milk of dairy cows (Bouwstra et al. 2008). The level of vitamin E provisioning afforded to the yolk is also likely to reflect that in the mother (Surai et al. 1999a; Surai 2000; Surai et al. 2016). Indeed, we found a negative correlation between yolk levels of MDA and α-tocopherol in 2010, suggesting

that mothers with relatively high levels of oxidative damage indicative of a poor oxidative state had relatively low levels of vitamin E. Therefore, females suffering higher levels of oxidative stress during reproduction, either due to greater investment or depleted resources of dietary antioxidants, may transfer the oxidative cost of reproduction to their offspring. This presents a trade-off between parent and offspring fitness as females choosing to invest more in reproduction may inadvertently damage offspring viability by incurring higher oxidative stress that is endowed to eggs.

Contrary to other findings (Surai & Speake 1998; Karadas et al. 2005; Bertrand et al. 2006), we found no influence of carotenoids on either baseline levels of yolk MDA or the susceptibility of yolk to peroxidation *in vitro*. This outcome supports recent scepticism of the functional role of carotenoids in antioxidant protection (Halliwell 1999; Casagrande et al. 2011; Costantini & Møller 2008; Costantini et al. 2010) particularly in wild birds which have shown huge variation in yolk carotenoid deposition. However, evidence of discrimination between different carotenoids by the avian embryo (Surai & Speake 1998) may indicate that carotenoid antioxidant function might be type-specific (Catoni et al. 2008; Isaksson et al. 2008) and employed at later stages of development, with limited activity in the freshly laid pre-embryonic eggs collected in this study.

The results presented here support the notion that the transmission of products of oxidative lipid damage to eggs may represent a transgenerational cost of reproduction (Blount et al. 2016). Furthermore, our results suggest a crucial protective role of maternally-derived vitamin E in egg yolk, independently of likely direct benefits of vitamin E for embryo and chick development. While oxidative stress remains a much debated physiological limitation of reproduction

(Isaksson et al. 2011; Metcalfe & Monaghan 2013; Selman et al. 2012; Speakman & Garratt 2014; Blount et al. 2016), our results raise the possibility that the oxidative status of breeding females may indeed influence offspring fitness via effects on egg oxidative state. Considering the importance of vitamin E, a dietary antioxidant, as a critical protective presence in yolk, further investigation into the environmental mediators of dietary antioxidants would greatly increase understanding of the relationship between oxidative stress, the antioxidant system and the transgenerational impacts of oxidative stress via yolk deposition in wild populations.

Chapter 4

General Discussion

Oxidative stress is considered to be a likely potential mechanism underpinning life history trade-offs but, thus far, empirical evidence supporting this theory has yielded equivocal support. Since the suggestion that oxidative stress could determine rates of senescence (Harman 1956; Finkel & Holbrook 2000; Halliwell 1999), there has been much interest in the role of oxidative stress in shaping life history trade-offs. Further knowledge about the role of oxidative stress and its functions led to the popular idea that it may provide the functional system that underpins proximate decisions about resource allocation, ultimately determining life history trade-offs; the most important of which being the trade-off between reproduction and longevity (i.e. the 'cost of reproduction'; Dowling & Simmons 2009; Metcalfe & Alonso-Alvarez 2010; Metcalfe & Monaghan 2013; Monaghan et al. 2009; Selman et al. 2012; Speakman & Garratt 2014). However, after several decades of investigation, empirical studies have failed to provide substantial evidence either in support or disagreement with the theory that there is an oxidative cost of reproduction.The lack of a universally convincing outcome has been attributed, firstly, to studies measuring antioxidants rather than actual markers of oxidative stress, and later to inconsistency in both study design and measurement of oxidative stress markers (Isaksson et al. 2011; Metcalfe & Monaghan 2013; Selman et al. 2012; Speakman & Garratt 2014; Blount et al. 2016).

In response to a flurry of review articles discussing the limitations of recent studies and suggesting areas of improvement (Isaksson et al. 2011; Selman et al. 2012; Stier et al. 2012; Metcalfe & Monaghan 2013; Speakman & Garratt

2014), a new hypothesis has been presented aiming to provide a coherent explanation for the conflicting results seen so far. The ‘oxidative shielding’ hypothesis was developed from a detailed meta-analysis of empirical studies testing the oxidative cost of reproduction across a range of taxa, incorporating results selected by specific criteria to create a framework for the hypothesis (Blount et al. 2016). The hypothesis suggests that reproduction has dual impacts on both mother and offspring via transmission of maternally derived oxidative damage to gametes and developing offspring that are particularly vulnerable to impacts of oxidative attack. Such fitness impacts on offspring are suggested to cause selection on mothers to diminish oxidative stress while reproducing, creating the shielding pattern observed in the meta-analysis. Oxidative shielding may, therefore, be the reason for such variation in results thus far, as the timing of sampling is evidently critical if oxidative status in females is changing over the reproductive process. Notably, the ‘oxidative shielding’ hypothesis emphasises the importance of maternally-derived oxidative stress on offspring fitness and the influence it may have in determining the evolution of reproductive strategies; a concept that future studies should consider.

Our data show that oxidative stress may indeed be critical for fitness via its influential role as a maternal effect in the production and survival of offspring. Thus far, the field of maternal effects research has been dominated by investigation into the benefits that they can provide to both parent and offspring (Marshall & Uller 2007). However, our data are among the first to show evidence of naturally occurring maternal effects with negative impacts. We have presented data that suggest that the transmission of oxidatively damaged lipids to eggs reduces fledgling survival, exposing a second oxidative cost of

reproduction borne by the offspring. Indeed, our data suggest that the level of vitamin E present in the yolk significantly influences the susceptibility of yolk lipids to peroxidation, implying that there may be a functional protective role of vitamin E in the pre-embryonic egg. Despite much focus on the associations between embryo development and antioxidant uptake (Surai et al. 1996; Surai 1999), the concept that antioxidants provided in the egg may afford oxidative protection in the early stages of incubation has not, to our knowledge, been examined. Interestingly, carotenoids were not observed to have any influence on yolk susceptibility to peroxidation, suggesting that in pre-embryonic yolk such as we measured, vitamin E is the main antioxidant providing protection against oxidative attack. Because eggs contain a limited range of antioxidants mainly comprising vitamin E, A and carotenoids, the functional role of these antioxidants is more clearly identified, promoting the use of simple yet specific experimental design for future studies.

The findings in this study suggest that the transmission of oxidative damage to offspring carries a negative fitness impact,which implies that there should be selection acting on females to produce high quality eggswith low peroxidised lipid transfer and high antioxidant status. Indeed, all females showed a reduction in levels of plasma vitamin E from pre-laying to clutch completion, likely caused by maternal investment of essential antioxidants in egg yolk (Royle et al. 2001; Perrins 1996; Surai 2000). Given that egg composition is entirely a function of maternal state, this suggests that in order to produce high quality eggs with minimal transfer of oxidative damage, there may be selection for maternal oxidative stress mitigation during the laying period. Our observation that maternal plasma levels of MDA converge towards a rather similar phenotype between pre-laying and clutch completion support this view. The

results presented in this thesis support the ‘oxidative shielding’ hypothesis in so far as we have witnessed for the first time in an avian species a decline in circulating levels of oxidative damage over the course of egg production, at least in some females, which is suggestive of the proposed shielding effect. Similar results were recently observed in reproducing female banded mongooses *Mungosmungo* that showed lower levels of circulating MDA during pregnancy compared with before and after (Vitikainen et al. 2016). In our study, the convergence of female plasma phenotype just after clutch completion provides a rare insight into the female physiological state immediately after egg production. However, our data do not provide conclusive evidence of shielding, merely an indication that there is a conformation of maternal oxidative phenotype at the point of laying. We therefore suggest that the ‘oxidative shielding’ hypothesis would benefit from further studies examining the longitudinal change in female oxidative status, ensuring a similarly specific study design.

The mechanisms that allow females to decrease levels of oxidative damage are unclear. Contrary to expectation, there was no observable relationship between pre-laying plasma levels of MDA and vitamin E, supporting the consensus that antioxidants alone are not good indicators of oxidative stress (Monaghan et al. 2009; Selman et al. 2012).We have considered carotenoids and tocopherols in this study because they are major families of lipophilic non-enzymatic antioxidants, and are the main antioxidants found within eggs. However, other types of antioxidants may be more influential in mitigating oxidative stress in the various tissues of reproducing females.Although there wasn’t a clear relationship between vitamin E and lipid peroxidation in our study, the decline in vitamin E from pre-laying to clutch completion may have been caused by rapid

depletion as tocopherols were employed to combat elevated ROS levels generated by egg production. A similar result was reported in zebra finches where reproduction was associated with a decline in total antioxidant defences (Alonso-Alvarez et al. 2004). However, levels of antioxidants may both increase and decrease under conditions of oxidative stress, so such results must be interpreted with caution (Blount et al. 2016; Selman et al. 2012).

Sex hormones such as oestrogen have also been seen to provide a mechanism for oxidative stress mitigation (Sugioka et al. 1987), such as in female rats where oestradiol supressed lipid peroxidation (Huh et al. 1994; Persky et al. 2000) and increased glutathione activity (Borrás et al. 2003). Alternatively, uncoupling proteins (UCPs) have also been proposed as a mechanism of minimising ROS emission from the electron transport chain by inducing proton leaks, generating a negative feedback loop to prevent further mitochondrial ROS production (Mailloux & Harper 2011; Brand 2000; Echtay 2002). However, UCPs reduce the generation of ROS by diverting energy away from ATP synthesis (Echtay 2002; Brand 2000), which is inefficient and therefore an unlikely mechanism for sustained oxidative damage mitigation. Indeed, gene expression of UCPs is downregulated in lactating small mammals (Speakman 2008), suggesting this is not a key mechanism in ROS control during reproduction, at least in this taxa. Research into the role of UCPs in avian studies has primarily focussed on their thermoregulatory role (Talbot et al. 2004; Raimbault et al. 2001) and therefore further research is necessary to gain a better understanding of the functional role of UCPs in mitigating avian oxidative stress.

If we accept that oxidative stress is indeed a crucial functional mechanism underpinning fundamental life history trade-offs, then the challenge for future research is to examine how changing environments and ecological pressures might influence life history trajectories via constraints and costs imposed by oxidative stress. It is possible that the persistence of deleterious MDA incorporation into egg yolk may be the result of an environmentally dependent variability of impact, whereby the costs and benefits of shielding change both seasonally and annually. There is likely to be a cost associated with oxidative shielding, which could manifest as a constraint on the number or quality of offspring produced, or as a subsequent elevation of oxidative stress for the female after reproduction. For example, the transmission of oxidative damage to offspring may have only mild impacts if antioxidant-rich food resources are abundant during the provisioning period, thereby offsetting oxidative damage to offspring. Indeed, a comparative avian study found that the availability and consumption of some dietary antioxidants in the environment were directly related to levels within the birds themselves, although interestingly, vitamin E was not observed to have any relationship with diet across thirteen of the bird species (Cohen et al. 2009). However, a cross-fostering experiment in Alpine swifts *Tachymarptis melba*found that eggs laid by mothers with lower susceptibility to oxidative stress were more likely to hatch, irrespective of their incubation environment (Bize et al. 2008), suggesting that egg quality is most important for chick fitness and therefore subsequent antioxidant provisioning may not be adequate to mitigate these early maternal effects. Further investigation into the relationship between markers of oxidative stress and the antioxidant system would advance understanding of their synergistic

relationship and how they work to maintain the oxidative balance during the reproductive process.

It is possible that parental oxidative status will be influenced by seasonal weather fluctuations that determine both metabolic rate and food availability. This in turn may have profound effects on egg quality if egg formation is dependent on available antioxidant resources, such as in income breeders. Furthermore, it is possible that mis-timing of egg laying and food abundance have significant implications for reproductive success (Winkler et al. 2002; Dunn 2004; Visser et al. 2004), particularly if it influences the availability of antioxidants required for oxidative protection within eggs. This draws attention to the need for further studies to focus on wild populations within a natural context, as environmental factors are likely to play an important role in determining the oxidative status of individuals, either directly or indirectly.

Other factors such as the experience of the female in early life may influence reproductive strategy in response to oxidative status. For example, female captive zebra finches from larger broods had lower erythrocyte resistance to oxidative stress, produced fewer eggs in each clutch, but experienced a longer reproductive lifespan, resulting in similar lifetime fecundity to females producing larger broods over a shorter reproductive lifespan (Alonso-Alvarez 2006). Although female experience is unknown in our study, it is worth considering that female oxidative status at the pre-laying period and her reproductive decisions are likely the product of both her own life experience and the environment in which she prepares for breeding (Bergeron et al. 2011; Limón-Pacheco & Gonsebatt 2009), cumulatively resulting in the oxidative status that may determine her reproductive investment. Additionally, it is possible that

resistance to oxidative stress can be genetically inherited, either directly or via epigenetic effects (Gluckman et al. 2008), but the influence of genes over maternal or environmental effects is likely to change at different developmental stages and is difficult to assess (Kim, Velando, et al. 2010; Kim, Noguera, et al. 2010). Certainly, species-specific life history strategies must be considered, as influences such as predation risk are likely to determine reproductive investment and the cost-benefit balance of incurring oxidative stress (Kirkwood & Holliday 1979; Selman et al. 2012). Therefore, there are likely to be multiple trade-offs as individuals modulate lifetime reproductive success depending on resource availability and individual quality, which may confound results from a single reproductive attempt (Metcalfe & Monaghan 2013).Reproductive manipulation and cross-fostering experiments would be instrumental in advancing our knowledge of the distinctions between the influences of maternal, environmental and additive genetic effects of oxidative stress on reproduction.

This study is among the first to investigate the oxidative cost of reproduction within the framework of the 'oxidative shielding' hypothesis. Empirical evidence provided in this study supports the concept that oxidative stress is an important functional mechanism that underpins reproductive decisions. We have observed oxidative stress both as a constraint on mothers, by mediating clutch size, and as a cost to fitness in terms of offspring survival. We recommend that future studies investigating the oxidative cost of reproduction should be designed utilising the framework presented by the 'oxidative shielding' hypothesis. Furthermore, study designs should ensure that both the timing of sampling and markers of oxidative stress are carefully specified to generate results that can be more clearly interpreted. Finally, studies should aim to further investigate the transgenerational impacts of oxidative damage, particularly how maternally-

derived oxidative stress in offspring might provide a crucial selection pressure driving key reproductive decisions, in a variety of taxa. There is much scope for further study to substantiate the hypothesis and lead the field of oxidative stress and reproduction research onto the next chapter.

References

Agarwal, A., Gupta, S., Sharma, R.K.(2005). Role of oxidative stress in female reproduction. *ReprodBiolEndocrinol*, 3.

Agarwal, R., Chase, S.D.(2002). Short communication Rapid, fluorimetric–liquid chromatographic determination of malondialdehyde in biological samples. *Journal of Chromatography B*, 775, pp.121–126.

Al-Gubory, K.H., Fowler, P.A., Garrel, C.(2010). The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. *Int J Biochem Cell Biol*, 42(10), pp.1634–1650.

Alonso-Alvarez, C.(2006). An experimental manipulation of life-history trajectories and resistance to oxidative stress. *Evolution*, 60(9), pp.1913–1924.

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Sorci, G.(2004). Increased susceptibility to oxidative stress as a proximate cost of reproduction. *EcologyLetters*, 7(5), pp.363–368.

Alonso-Álvarez, C., Pérez-Rodríguez, L., García, J., Viñuela, J., Mateo, R.(2010). Age and breeding effort as sources of individual variability in oxidative stress markers in a bird species. *Physiological and Biochemical Zoology*, 83(1), pp.110–118.

Ayala, A., Muñoz, M.F., Argüelles, S.(2014). Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*. Article ID 360438.

Balaban, R.S., Nemoto, S., Finkel, T.(2005). Mitochondria, Oxidants and ageing. *Cell,* 120(4), pp.483–495.

Barja, G.(2007). Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: implications for aging studies. *Rejuvenation research,* 10,(2), pp.215–224.

Beckman, K.B., Ames, B.N.(1998). The Free Radical Theory of Aging Matures. *Physiological Reviews*, 78(2), pp.547–581.

Bergeron, P., Careau, V., Humphries, M. M., Réale, D., Speakman, J. R., Garant, D. (2011). The energetic and oxidative costs of reproduction in a free-ranging rodent. *Functional Ecology*, 25(5), pp.1063–1071.

Bernardo, J.(1996). Maternal Effects in Animal Ecology 1. *Amer. Zool*., 36(2), pp.83–105.

Bertrand, S., Alonso-Alvarez, C., Devevey, G., Faivre, B., Prost, J., Sorci, G.(2006). Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. *Oecologia,* 147(4), pp.576–584.

Bize, P., Devevey, G., Monaghan, P., Doligez, B., Christe, P.(2008). Fecundity and survival in relation to resistance to oxidative stress in a free-living bird. *Ecology*, 89(9), pp.2584–2593.

Blount, J.D., Surai, P. F., Nager, R. G., Houston, D. C., Møller, A. P., Trewby M. L., Kennedy, M. W.(2002). Carotenoids and egg quality in the lesser blackbacked gull Larusfuscus: a supplemental feeding study of maternal effects*. Proceedings. Biological sciences / The Royal Society*, 269(1486), pp.29–36.

Blount, J.D., Vitikainen, E. I. K., Stott, I., Cant, M. A.(2016). Oxidative shielding and the cost of reproduction. *Biological Reviews*, 44, pp.483–497.

Blount, J.D., Houston, D.C.,Moller, A.P. (2000). Why egg yolk is yellow. *Trends inEcology and Evolution*, 15(2), pp.47–49.

Borrás, C., Sastre, J., García-Sala, D., Lloret, A., Pallardó, F., Viña, J.(2003). Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radical Biology and Medicine*, 34(5), pp.546–552.

Bouwstra, R.J., Goselink, R. M. A., Dobbelaar, P., Nielen, M., Newbold, J. R., van Werven, T.(2008). The Relationship Between Oxidative Damage and Vitamin E Concentration in Blood, Milk, and Liver Tissue from Vitamin E Supplemented and NonsupplementedPeriparturient Heifers. *Journal of Dairy Science*, 91(3), pp.977–987.

Brand, M.D. (2000). Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Experimental Gerontology*, 35, pp.811–820.

Burger, J., Eichhorst, B.(2005). Heavy metals and selenium grebe eggs from Agassiz National Wildlife refuge in nothern Minnesota. *Environmental Monitoring and Assessment,* 107(1–3), pp.285–295.

Burton, G.(1994). Vitamin E: Molecular and biological function. *Proceedings of the Nutrition Society,* 53(2), pp.251–262.

Casagrande, S., Dell'Omo, G., Constantini, D., Tagliavini, J., Groothuis, T. (2011). Comparative Biochemistry and Physiology , Part A Variation of a carotenoid-based trait in relation to oxidative stress and endocrine status during the breeding season in the Eurasian kestrel : A multi-factorial study. *Comparative Biochemistry and Physiology, Part A,* 160(1), pp.16–26.

Catoni, C., Peters, A.,Martin Schaefer, H.(2008). Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. *Animal Behaviour,* 76(4), pp.1107–1119.

Chatelain, M., Gasparini, J., Haussy, C., Frantz, A.(2016). Trace Metals Affect Early Maternal Transfer of Immune Components in the Feral Pigeon*. Physiological and Biochemical Zoology,* 89(3), pp.206–212.

Cohen, A.A., McGraw, K.J., Robinson, W.D.(2009). Serum antioxidant levels in wild birds vary in relation to diet, season, life history strategy, and species*. Oecologia,* 161(4), pp.673–683.

Costantini, D.,Dell'Omo, G.(2006). Environmental and genetic components of oxidative stress in wild kestrel nestlings (Falco tinnunculus). *Journal of Comparative*

Physiology B: Biochemical, Systemic, and Environmental Physiology, 176(6), pp.575–579.

Costantini, D., Casasole, G., AbdElgawad, H., Asard, H., Eens, M. (2016). Experimental evidence that oxidative stress influences reproductive decisions. *Functional Ecology*, 30(7), pp.1169–1174.

Costantini, D., Rowe, M., Butler, M. W., McGraw, K. J.(2010). From molecules to living systems: historical and contemporary issues in oxidative stress and antioxidant ecology. *Functional Ecology*, 24(5), pp.950–959.

Costantini, D., Bonisoli-alquati, A., Rubolini, D., Caprioli, M., Ambrosini, R., Saino, N.(2014). Nestling rearing is antioxidant demanding in female barn swallows (Hirundorustica). *Naturwissenschaften*, 101, pp.541–548.

Costantini, D.(2008). Oxidative stress in ecology and evolution: lessons from avian studies. *Ecology Letters*, 11(11), pp.1238–1251.

Costantini, D., Casasole, G., Eens, M.(2014). Does reproduction protect against oxidative stress? *Journal of Experimental Biology,* 217, pp.4237–4243.

Costantini, D.,Møller, A.P. (2008). Carotenoids are minor antioxidants for birds. *Functional Ecology,* 22(2), pp.367–370.

De Coster, G., De Neve, L., Verhulst, S., Lens, L. (2012). Maternal effects reduce oxidative stress in female nestlings under high parasite load. *Journal of Avian Biology,* 43(2), pp.177–185.

Cram, D.L., Blount, J. D., Young, A. J.(2015.) Oxidative status and social dominance in a wild cooperative breeder. *Functional Ecology*, 29(2), pp.229–238.

Crespi, B., Semeniuk, C.(2004). Parent-offspring conflict in the evolution of vertebrate reproductive mode. *Am Nat*, 163(5), pp.635–53.

D'Alba, L., Shawkey, M. D., Korsten, P., Vedder, O., Kingma, S. A., Komdeur, J., Beissinger, S. R.(2010). Differential deposition of antimicrobial proteins in blue tit (Cyanistes caeruleus) clutches by laying order and male attractiveness. *Behavioral Ecology and Sociobiology*, 64(6), pp.1037–1045.

Davies, K.J.A.(2000). Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB Life*, 50(4–5), pp.279–289.

Dowling, D.K., Simmons, L.W.(2009). Reactive oxygen species as universal constraints in life-history evolution*. Proceedings. Biological sciences / The Royal Society*, 276(1663), pp.1737–45.

Dunn, P.O.(2004). Breeding Dates and Reproductive Performance. *Advances in Ecological Research,* 35, pp.69–87.

Echtay, K.S.(2002). Superoxide activates mitochondrial uncoupling proteins. *Nature*, 415, pp.96–99.

Finkel, T., Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, 408(6809), pp.239–247.

Fletcher, Q.E., Selman, C., Boutin, S., McAdam, A. G., Woods, S. B., Seo, A. Y., Leeuwenburgh, C., Speakman, J. R., Humphries, M. H.(2012). Oxidative damage increases with reproductive energy expenditure and is reduced by food-supplementation. *Evolution,* 67(5), pp.1527–1536.

Garratt, M., Vasilaki, A., Stockley, P., McArdle, F., Jackson, M., Hurst, J. L.(2011). Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. *Proceedings of the Royal Society B,* 278(1708), pp.1098–1106.

Garratt, M., Pichaud, N., King, E. D. A., Brooks, R. C.(2013). Physiological adaptations to reproduction. I. Experimentally increasing litter size enhances aspects of antioxidant defence but does not cause oxidative damage in mice. *The Journal of experimental biology*, 216(Pt 15), pp.2879–88.

Garratt, M., McArdle, F., Stockley, P., Vasilaki, A., Beynon, R. J., Jackson, M. J., Hurst, J. L.(2012). Tissue-dependent changes in oxidative damage with male reproductive effort in house mice. *Functional Ecology*, 26(2), pp.423–433.

Gluckman, P.D., Hanson, M. A., Cooper, C., Thornburg, K. L.(2008). Effect of In Utero and Early-Life Conditions on Adult Health and Disease.*The New England Journal of Medicine,* 359, pp.61–73.

Groothuis, T.G.G, Schwabl, H.(2008). Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 363(1497), pp.1647–1661.

Groothuis, T.G.G., Müller, W., von Engehardt, N., Carere, C., Eising, C.(2005). Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neuroscience and Biobehavioral Reviews*, 29(2), pp.329–352.

Grune, T.,Krämer, Hoppe, P. P., Siems, W.(2001). Enrichment of eggs with n−3 polyunsaturated fatty acids: Effects of vitamin E supplementation. *Lipids*, 36(8), pp.833–838.

Gutteridge, J.M.C., Halliwell, B.(2000). Free Radicals and Antioxidants in the Year 2000 A Historical Look to the Future. *Annals of the New York Academy of Sciences*. 899, pp.136–147.

Hall, M.E., Blount, J. D., Forbes, S., Royle, N. J.(2010). Does oxidative stress mediate the trade-off between growth and self-maintenance in structured families? *Functional Ecology*, 24(2), pp.365–373.

Halliwell, B. (1999). Antioxidant defence mechanisms : From the beginning to the end (of the beginning).*Free Radic Res*, 31, pp.261–272.

Halliwell, B.(1991). Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. *The American Journal of Medicine*, 91(3), pp.S14–S22.

Haq, A.U., Bailey, C.A., Chinnah, A.(1996). Effect of beta-carotene, canthaxanthin, lutein, and vitamin E on neonatal immunity of chicks when supplemented in the broiler breeder diets. *Poultry science*, 75(9), pp.1092–7.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *Journal of gerontology*, 11(3), pp.298–300.

Hartley, R.C., Kennedy, M.(2004). Are carotenoids a red herring in sexual display? *Trends in Ecology and Evolution*, 19(7), pp.353–354.

Hayward, L.S., Wingfield, J.C.(2004). Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. *General and Comparative Endocrinology*, 135(3), pp.365–371.

Heaney, A. V., Monaghan, P.(1995). A Within-Clutch Trade-Off between Egg Production and Rearing in Birds. *Proceedings of the Royal Society B*, 261(1362), pp.361–365.

Huh, K., Shin, U. S., Choi, J. W., Lee, S. I. (1994). Effect of sex hormones on lipid peroxidation in rat liver. *Archives of Pharmacal Research*, 17(2), pp.109–114.

Hulbert, A.J., Pamplona, R., Buffstein, R., Buttemer, W. A.(2007). Life and Death : Metabolic Rate, Membrane Composition, and Life Span of Animals*. Physiological Reviews,* pp.1175–1213.

Isaksson, C.(2013). Opposing effects on glutathione and reactive oxygen metabolites of sex, habitat, and spring date, but no effect of increased breeding density in great tits (Parus major). *Ecology and Evolution*, 3(8), pp.2730–2738.

Isaksson, C., While, G. M., Olsson, M., Komdeur, J., Wapstra, E. (2011). Oxidative stress physiology in relation to life history traits of a free-living vertebrate: the spotted snow skink, Niveoscincusocellatus. *IntegrZool*, 6(2), pp.140–149.

Isaksson, C.(2009). The Chemical Pathway of Carotenoids: From Plants to Birds. *Ardea*, 97(1), pp.125–128.

Isaksson, C., Johansson, A., Andersson, S.(2008). Egg yolk carotenoids in relation to habitat and reproductive investment in the great tit Parus major*. Physiological and biochemical zoology*, 81(1), pp.112–8.

Isaksson, C., Sheldon, B.C., Uller, T.(2011). The Challenges of Integrating Oxidative Stress into Life-history Biology. *BioScience*, 61(3), pp.194–202.

Karadas, F., Pappas, A. C., Surai, P. F., Speake, B. K.(2005). Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 141(2), pp.244–251.

Kim, S.Y., Velando, A., Sorci, G., Alonso-Alvarez, C. (2010). Genetic correlation between resistance to oxidative stress and reproductive life span in a bird species. *Evolution,* 64(3), pp.852–857.

Kim, S.Y., Noguera, J.C., Morales, J., Velando, A. (2010). Heritability of resistance to oxidative stress in early life. *J ofEvolBiol*, 23(4), pp.769–775.

Kirkwood, T.B.L., Holliday, R.(1979). The Evolution of Ageing and Longevity. *Proceedings of the Royal Society of London B. Biological Sciences*, 205(1161), p.531 LP-546.

Kubota, R., Kunito, T., Tanabe, S., Ogi, H., Shibata, Y. (2002). Maternal transfer of arsenic to eggs of black-tailed gull (Laruscrassirostris) from Rishiri Island, Japan. *Applied Organometallic Chemistry*, 16(8), pp.463–468.

Limón-Pacheco, J., Gonsebatt, M.E.(2009). The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 674(1–2), pp.137–147.

Lin, Y. F., Tsai, H. L., Lee, Y. C., Chang, S. J.(2005). Maternal vitamin E supplementation affects the antioxidant capability and oxidative status of hatching chicks. *The Journal of Nutrition*, 135(10), pp.2457–61.

Losdat, S., Helfenstein, F., Blount, J. D., Richer, H.(2014). Resistance to oxidative stress shows low heritability and high common environmental variance in a wild bird. *Journal of Evolutionary Biology,* 27(9), pp.1990–2000.

Mailloux, R.J., Harper, M.E. (2011). Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free radical biology & medicine,* 51(6), pp.1106–15.

Marshall, D.J. &Uller, T.(2007). When is a maternal effect adaptive? *Oikos*, 116(12), pp.1957–1963.

Mausseau, T. A. & Fox, C.W.(1998). The adaptive significance of maternal effects. *TREE Reviews*, 13(10), pp.403–407.

McGraw, K.J., Adkins-Regan, E., Parker, R.S.(2005). Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. *Naturwissenschaften,* 92(8), pp.375–380.

Merkling, T., Blanchard, P., Chastel, O., Glauser, G., Vallat-Michel, A., Hatch, S. A., Danchin, E., Helfenstein, F.(2017). Reproductive effort and oxidative stress: effects of offspring sex and number on the physiological state of a long-lived bird. *Functional Ecology,* 31(6), pp.1201–1209.

Metcalfe, N.B., Alonso-Alvarez, C. (2010). Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Functional Ecology,* 24(5), pp.984–996.

Metcalfe, N.B., Monaghan, P.(2013). Does reproduction cause oxidative stress? An open question. *Trends in Ecology and Evolution*, 28(6), pp.347–350.

Mohiti-Asli, M., Shariatmadari, F., Lotfollahian, H., Mazuji, M. T.(2008). Effects of supplementing layer hen diets with selenium and vitamin E on egg quality, lipid oxidation and fatty acid composition during storage. *Canadian Journal of Animal Science,* 88(3), pp.475–483.

Monaghan, P., Bolton, M., Houston, D.C.(1995). Egg production constraints and the evolution of avian clutch size. *Proceedings of the Royal Society B: Biological Sciences,* 259, pp.189–191.

Monaghan, P., Metcalfe, N.B., Torres, R.(2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology Letters*, 12(1), pp.75–92.

Monaghan, P., Nager, R.G.(1997). Why don't birds lay more eggs? *Trends in Ecology and Evolution*, 12(7), pp.270–274.

Monaghan, P., Nager, R.G., Houston, D.C.(1998). The price of eggs: increased investment in egg production reduces the offspring rearing capacity of parents. *Proceedings of the Royal Society B: Biological Sciences,* 265(1407), pp.1731–1735.

Mora, M.A. (2003). Heavy metals and metalloids in egg contents and eggshells of passerine birds from Arizona. *Environmental Pollution*, 125(3), pp.393–400.

Mourente, G., Tocher, D. R., Diaz, E., Grau, A., Pastor, E.(1999). Relationships between antioxidants, antioxidant enzyme activities and lipid peroxidation products during early development in Dentexdentex eggs and larvae. *Aquaculture*, 179(1–4), pp.309–324.

Muriel, J., Salmón, P., Nunez-Buiza, A., de Salas, F., Pérez-Rodríguez, L., Puerta, M., Gil, D. (2015). Context-dependent effects of yolk androgens on nestling growth and immune function in a multibrooded passerine. *J of EvolBiol,* 28(8), pp.1476–1488.

Nager, R.G., Monaghan, P., Houston, D.C.(2000). Within-clutch trade-offs between the number and quality of eggs: Experimental manipulations in gulls. *Ecology*, 81(5), pp.1339–1350.

Nilsson, J.Å., Råberg, L. (2001). The resting metabolic cost of egg laying and nestling feeding in great tits. *Oecologia*, 128(2), pp.187–192.

Nussey, D.H. et al.(2009). Life history correlates of oxidative damage in a free-living mammal population. *Functional Ecology*, 23(4), pp.809–817.

Ołdakowski, Ł.,Piotrowska, Ż, Chrząścik, K. M., Sadowska, E. T., Koteja, P, Taylor, J. R. E.(2012). Is reproduction costly? No increase of oxidative damage in breeding bank voles. *The Journal of Experimental Biology*, 215(11), pp.1799–1805.

Olsson, M., Wilson, M. Uller, T., Mott, B., Isaksson, C.(2009). Variation in levels of reactive oxygen species is explained by maternal identity, sex and body-size-corrected clutch size in a lizard. *Naturwissenschaften*, 96(1), pp.25–29.

Parolini, M.,Khoriauli, L., Possenti, C. D., Colombo, G., Caprioli, M., Santagostino, M., Nergadze, S. G., Milzani, A., Giulotto, E., Saino, N.(2017). Yolk vitamin E prevents oxidative damage in gull hatchlings. *Royal Society Open Science*, 4, p.170098.

Perrins, C.M.(1979.) British tits, London: HarperCollins.

Perrins, C.M.(1996). Eggs, egg formation and the timing of breeding. *Ibis*, 138, pp.2–15.

Persky, A.M., Green, P. S., Stubley, L., Howell, C. O., Zaulyanov, L., Brazeau, G. A., Simpkins, J. W.(2000). Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro. *Proceedings of the Society for Experimental Biology and Medicine*, 223(1), pp.59–66.

Pihlaja, M., Siitari, H., Alatalo, R. V. (2006). Maternal antibodies in a wild altricial bird: Effects on offspring immunity, growth and survival. *Journal of Animal Ecology,* 75(5), pp.1154–1164.

Plummer, K.E., Bearhop, S., Leech, D. I., Chamberlain, D. E., Blount, J. D.(2013). Fat provisioning in winter impairs egg production during the following spring: A landscape-scale study of blue tits. *Journal of Animal Ecology*, 82(3), pp.673–682.

Plummer, K.E.(2011). The effects of over-winter dietary provisioning on health and productivity of garden birds., (June), PhD Thesis.

Raimbault, S.Dridi, S. ,Denjean, F., Lachuer, J., Couplan, E., Bouillaud, F., Bordas, A., Duchamp, C., Taouis, M., Ricquier, D.l.(2001). An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds. *The Biochemical journal,* 353(Pt 3), pp.441–4.

Räsänen, K., Kruuk, L.E.B., (2007). Maternal effects and evolution at ecological time-scales*. Functional Ecology*, 21(3), pp.408–421.

Del Rio, D., Stewart, A.J., Pellegrini, N., (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism and Cardiovascular Diseases*, 15(4), pp.316–328.

Rizzo, A., Pantaleo, M., Mutinati, M., Minoia, G., Trisolini, C., Ceci, E., Sciorsci, R.L. (2013). Blood and milk oxidative status after administration of different antioxidants during early postpartum in dairy cows. *Research in Veterinary Science,* 95(3), pp.1171–1174.

Royle, N.J.*,* Surai, P.F., McCartney, R.J., Speake, B.K. (1999). Parental investment and egg yolk lipid composition in gulls. *Functional Ecology*, 13(3), pp.298–306.

Royle, N.J., Surai, P.F., Hartley, I.R., (2001). Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects? *Behavioral Ecology,* 12(4), pp.381–385.

Rubolini, D., Romano, M., Boncoraglio, G., Ferrari, R.P., Martinelli, R., Galeotti, P., Fasola, M., Saino, N. (2005). Effects of elevated egg corticosterone levels on behavior, growth, and immunity of yellow-legged gull (Larusmichahellis) chicks. *Hormones and Behavior*, 47(5), pp.592–605.

Saino, N., Dall'Ara, P., Martinelli, R., Moller, A.P. (2002). Early maternal effects and antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. *Journal of Evolutionary Biology,* 15(5), pp.735–743.

Selman, C., Blount, J.D., Nussey, D.H., Speakman, J.R. (2012). Oxidative damage, ageing, and life-history evolution: where now? *Trends in Ecology & Evolution*, 27(10), pp.570–577.

Speakman, J.R., Blount, J.D., Bronikowski, A.M., Buffenstein, R., Isaksson, C., Kirkwood, T.B.L., Monaghan, P., Ozanne, S.E., Beaulieu, M., Briga, M., Carr, S.K., Christensen, L.L., Cochemé, H.M., Cram, D.L., Dantzer, B., Harper, J.M., Jurk, D., King, A., Noguera, J.C., Salin, K., Sild, E., Simons, M.J.P., Smith, S., Stier, A., Tobler, M., Vitikainen, E., Peaker, M., Selman, C. (2015). Oxidative stress and life histories: unresolved issues and current needs. *Ecology and Evolution,* 5(24), pp.5745–5757.

Speakman, J.R., (2008). The physiological costs of reproduction in small mammals. , *Philosophical Transactions of the Royal Society of London B.* 363.pp.375–398.

Speakman, J.R., Garratt, M., (2014). Oxidative stress as a cost of reproduction: Beyond the simplistic trade-off model. *BioEssays,* 36(1), pp.93–106.

Stearns, S.C.(1992). The evolution of life histories,*Zoology.* 1. pp. 249

Surai, P.F.(2000). Effect of selenium and vitamin E content of the maternal diet on the antioxidant system of the yolk and the developing chick. *British Poultry Science*, 41, pp.235–243.

Van den Steen, E., Jaspers, V.L.B., Covaci, A., Neels, H., Eens, M., Pinxten, R. (2009). Maternal transfer of organochlorines and brominated flame retardants in blue tits (Cyanistes caeruleus). *Environment International*, 35(1), pp.69–75.

Stier, A., Reichert, S., Massemin, S., Bize, P., Criscuolo, F.(2012). Constraint and cost of oxidative stress on reproduction: correlative evidence in laboratory mice and review of the literature. *Front Zool*, 9(1), pp.37.

Sugioka, K., Shimosegawa, Y., Nakano, M., (1987). Estrogens as natural antioxidants of membrane phospholipid peroxidation. *FEBS Letters*, 210(1), pp.37–39.

Surai, P.F.(2000). Effect of selenium and vitamin E content of the maternal diet on the antioxidant system of the yolk and the developing chick. *British Poultry Science*, 41(2), pp.235–243.

Surai, P.F.(2002). Natural antioxidants in avian nutrition and reproduction, Nottingham: Nottingham University Press.

Surai, P.F., (1999). Tissue-specific changes in the activities of antioxidant enzymes during the development of the chicken embryo. *British Poultry Science*, 40(3), pp.397–405.

Surai, P.F., Fisinin, V.I., Karadas, F., (2016). Antioxidant systems in chick embryo development. Part 1. Vitamin E, carotenoids and selenium. *Animal Nutrition,* 2, pp.1–11.

Surai, P.F., Noble, R.C., Speake, B.K. (1999a). Relationship between vitamin E content and susceptibility to lipid peroxidation in tissues of the newly hatched chick. *British Poultry Science*, 40(3), pp.406–410.

Surai, P.F., Noble, R.C., Speake, B.K. (1999b). Relationship between vitamin E content and susceptibility to lipid peroxidation in tissues of the newly hatched chick. *British poultry science*, 40(3), pp.406–10.

Surai, P.F., Noble, R.C., Speake, B.K.(1996). Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embryo. *Biochimica et BiophysicaActa - Lipids and Lipid Metabolism*. 1304. pp.1-10.

Surai, P.F., Speake, B.K., (1998). Distribution of carotenoids from the yolk to the tissues of the chick embryo. *The Journal of Nutritional Biochemistry*, 9(11), pp.645–651.

Suriyasathaporn, W., Vinitketkumnuen, U., Chewonarin, T., Chupia, V., Pinyopummintr, T.W. (2009). The indicative influence of oxidative stress on low milk yields in dairy cattle. *Thai Journal of Veterinary Medicine*, 39(3), pp.237–243.

Svensson, L., (1992). Identification guide to European passerines, Stockholm: NaturhistoriskaRiksmuseet.

Talbot, D.A., Duchamp, C., Rey, B., Hanuise, N., Rouanet, J.L., Sibille, B., Brand, M.D.(2004). Uncoupling protein and ATP/ADP carrier increase mitochondrial proton conductance after cold adaptation of king penguins. *The Journal of physiology*, 558(Pt 1), pp.123–135.

Tilgar,V.*,* Mägi, M., Lind, M., Lodjak, J., Moks, K., Mänd, R. (2016). Acute embryonic exposure to corticosterone alters physiology, behaviour and growth in nestlings of a wild passerine. *Hormones and Behavior*, 84, pp.111–120.

Toledo, A., Andersson, M.N., Wang, H-L., Salmón, P., Watson, H., Burdge, G.C., Isaksson, C. (2016). Fatty acid profiles of great tit (Parus major) eggs differ between urban and rural habitats, but not between coniferous and deciduous forests*. Naturwissenschaften*. 103(55).

Tschirren, B., Postma, E., Gustafsson, L., Groothuis, T.G.G., Doligez, B.(2014). Natural selection acts in opposite ways on correlated hormonal mediators of prenatal maternal effects in a wild bird population. *Ecology Letters*, 17(10), pp.1310–1315.

Verreault, J., Bech, C., Letcher, R.J., Ropstad, E., Dahl, E., Gabrielsen, G.W. (2007). Organohalogen contamination in breeding glaucous gulls from the Norwegian Arctic: Associations with basal metabolism and circulating thyroid hormones. *Environmental Pollution,* 145(1), pp.138–145.

Visser, M.E., Both, C., Lambrechts, M.M. (2004). Global Climate Change Leads to Mistimed Avian Reproduction. *Advances in Ecological Research*, 35, pp.89–110.

Visser, M.E., Lessells, C.M.(2001). The costs of egg production and incubation in great tits (Parus major). *Proceedings of the Royal Society B-Biological Sciences*, 268(1473), pp.1271–1277.

Vitikainen, E.I.K.,Cant, M.A., Sanderson, J.L., Mitchell, C., Nichols, H.J., Marshall,H., Thompson, F.J., Gilchrist, J.S., Hodge, S.J., Johnstone, R.A., Blount, J.D. (2016).

Evidence of Oxidative Shielding of Offspring in a Wild Mammal. *Frontiers in Ecology and Evolution,* 4(May), pp.1–10.

Wakefield, S.L., Lane, M., Schulz, S.J., Hebart, M.L., Thompson, J.G., Mitchell, M. (2008). Maternal supply of omega- 3 polyunsaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse. *Am. J. Physiol.-Endocrinol. Metab*., 294(2), pp.E425–E434.

Wiersma, P., Selman, C., Speakman, J.R., Verhulst, S. (2004). Birds sacrifice oxidative protection for reproduction. *Biology Letters.* 271. pp.360–363.

Winkler, D.W., Dunn, P.O., McCulloch, C.E. (2002). Predicting the effects of climate change on avian life-history traits. *Proceedings of the National Academy of Sciences of the United States of America*, 99(21), pp.13595–9.

Wolf, J.B., Brodie, E.D. (1998). The Coadaptation of Parental and Offspring Characters. *Evolution*. 52(2). pp.299–308.

Wolf, J.B., Wade, M.J., (2009). What are maternal effects (and what are they not)? *Philosophical Transactions of the Royal Society of London B*, 364(1520), pp.1107–1115.

Xu, Y.-C.*,* Yang, D-B., Speakman, J.R., Wang, D-H., Blount, J.D. (2014). Oxidative stress in response to natural and experimentally elevated reproductive effort is tissue dependent. *Functional Ecology*, 28(2), pp.402–410.

Yang, D.-B., Xu, Y-C., Wang, D-H., Speakman, J.R. (2013). Effects of reproduction on immuno-suppression and oxidative damage, and hence support or otherwise for their roles as mechanisms underpinning life history trade-offs, are tissue and assay dependent. *The Journal of experimental biology*, 216, pp.4242–4250.